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PRESERVATION OF CYANOGENETIC PLANTS FOR CHEMICAL ANALYSIS[1]

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INTRODUCTION

The need of an efficient preservative for samples of fresh cyanogenetic plants intended for chemical analysis has long been recognized. In the past, reliable figures for the hydrocyanic acid content of such plants could be obtained only from specimens collected immediately before analysis. This fact is due to the rapidity with which the hydrocyanic acid content changes after the plants are gathered, resulting in a variable loss of hydrocyanic acid which renders the analytical figures uncertain. Considerable disappearance of hydrocyanic acid occurring when cyanogenetic plants are stored with water has been reported by Dezani, (*13*)[2] Alsberg and Black, (*3*) and others (*20*, *26*). These considerations make impossible a comparison of plants grown in different places under different climatic and soil conditions if the samples must be transported great distances for analysis.

In a projected study of the factors involved in cyanogenesis it was desired to obtain samples of several varieties of cyanogenetic plants from widely scattered regions. These included localities in Utah, Colorado, Oklahoma, Texas, and Virginia. The chemical studies were conducted in the laboratories of the Bureau of Animal Industry in Washington, D. C. The transportation of some of the samples required 7 days by express, so that a long interval elapsed between collection and analysis. In some cases it appeared that refrigeration would preserve the samples without great change during transit, but this method was impracticable. It was decided, therefore, to study other means as well as refrigeration for preservation of the samples, and this paper records the results of that investigation.

FORMATION AND PRESERVATION OF HYDROCYANIC ACID IN PLANTS

Hydrocyanic acid does not appear to exist as such in cyanogenetic plants (*9*, *17*, *18*, *21*) but results from the interaction of an enzyme ("emulsin") and a glucoside which contains a CN group capable of being hydrolyzed to hydrocyanic acid. The rate of hydrocyanic acid formation, once the process begins, is originally rapid but diminishes after some hours and then proceeds slowly for an indeterminate time. Studies by Auld (*4*, *5*), Caldwell and Courtauld (*8*), Tammann (*24*), and Willstätter and Csányi (*31*) indicate that the enzymic hydrolysis of amygdalin is not complete. Auld observed a decomposition of 93.3 percent in 22 hours, and Caldwell and Courtauld report 98.2 and 98.5

[1] Received for publication March 8, 1938; issued August 1938.

[2] Italic numbers in parentheses refer to Literature Cited, p. 105.

percent in 67 and 90 hours, respectively. Auld considers these percentages too high.

Charlton (*9*) and, independently, Warth (*28*) found that after enzymic hydrolysis of Burma beans a further quantity of hydrocyanic acid could be obtained by extraction with hot water and acid hydrolysis of the extract. Narasimha Acharya (*1*) states that 72 hours is required for complete cyanogenesis in sorghum. Under these conditions it is essential that a preservative should not inactivate the enzyme present in the plant and so stop all cyanogenesis, and in addition that it should so act as to prevent or at least minimize loss of the hydrocyanic acid liberated by enzyme action. The ideal preservative would be one that could act solely to prevent loss of hydrocyanic acid without disturbing other metabolic processes in the sample, and that would permit quantitative recovery of the hydrocyanic acid produced during the preservation.

Some attempts to obtain an effective preservative have been reported. Brünnich (*7*) stated that the addition of formalin or chloroform to sorghum completely stops formation of hydrocyanic acid. Alsberg and Black (*2*) employed alcohol to preserve specimens of certain grasses. Willaman and West (*30*) used 20 cc of 3-percent alcoholic sodium hydroxide and 2 cc of chloroform for samples preserved for 4 to 8 days and reported no loss of hydrocyanic acid in the material. Willaman (*29*) in a later study found alcohol and mixtures of alcoholic sodium hydroxide with chloroform and of alcohol with chloroform or ether to be effective preservatives of sorghum. He concluded that alcohol, chloroform, and ether stimulate the synthetic action of the glucoside enzymes, leading to an increased hydrocyanic acid content of the plant.

BACTERIAL DECOMPOSITION

In the storage of plant material the question of the possibility and effect of bacterial decomposition arises. The observation that certain micro-organisms may so decompose protein as to lead to the formation of hydrocyanic acid introduces a factor that must be considered in all studies on preservation. Emerson, Cady, and Bailey (*14*) report that certain micro-organisms living on slightly acid protein media evolve hydrocyanic acid provided no free mineral acid is present. Clawson and Young (*10*) identified one of these organisms as *Bacillus pyocyaneus* and stated that *B. fluorescens* and other organisms produce hydrocyanic acid from proteins. Clawson and Young call attention to the fact that in most of the work that has been done on cyanogenetic plants the workers were using nonsterile material "which could have been easily contaminated by a hydrocyanic acid-producing organism."

The probability that this explanation of cyanogenesis would account for the hydrocyanic acid content reported for *Phaseolus lunatus*, sorghum, wild cherry, flax, and other cyanogenetic plants was removed by the isolation of the cyanophoric glucosides present in these plants and the proof that they may evolve hydrocyanic acid under sterile conditions such as boiling with strong mineral acids.

Further evidence on this point is afforded by the present study in which it has been found that cyanogenetic plants preserved with 1 and 2 percent of mercuric chloride evolved as much and in some cases more hydrocyanic acid than control plants stored under nonsterile conditions.

PLANTS USED IN THIS INVESTIGATION

A number of different species of cyanogenetic plants were used in this study since it was not known whether an effective preservative for one type of plant would be equally effective for a different species. In most cases both fresh and dried material was available, but for Johnson grass (*Sorghum halepense*) only dried plant was obtained. Wild cherry (*Prunus serotina*) was collected in Arlington County, Va., as needed. Another species, *P. melanocarpa*, was collected in the neighborhood of Salina, Utah, by A. B. Clawson, of the Bureau of Animal Industry, and shipped to the laboratory both as dried plant and in preservative. Arrowgrass (*Triglochin maritima*) was also collected by Clawson in various localities in Utah and was shipped both as dried plant and in preservative. Sudan grass (*S. vulgare* var. *sudanensis*) was collected near Denver, Colo., by G. W. Stiles, Jr., of this Bureau, and was shipped both as green and as dried plant. Dried Johnson grass was obtained from Woodward, Okla., through the Bureau of Dairy Industry. A large number of specimens of sorghum (*S. vulgare*) were obtained from a variety of sources. Through the courtesy of J. H. Martin, of the Bureau of Plant Industry, growing plants of 15 varieties of sorghum were made available, and later plants growing under greenhouse conditions were provided. Dried material of 12 varieties of sorghum grown at Chillicothe, Tex., was obtained through the courtesy of J. C. Stephens, of the Bureau of Plant Industry. The principal studies of sorghum were made with the varieties hegari, spur feterita, and white Italian broomcorn. Specimens of green arrowgrass and *P. melanocarpa* were preserved by Clawson in Utah before shipment. All other specimens used in this study were preserved in the laboratory at Washington, D. C.

METHOD OF PREPARING AND PRESERVING SPECIMENS

Customarily 100 g of fresh plant was taken. The quantity of dried plant used was 10, 25, or 50 g, depending on the cyanide content of the specimen. The dried plant was ground through a food chopper or mill, placed in a pint-sized clamp-topped preservative jar equipped with a glass top and rubber gasket, the preservative was added, and, except in special cases, the jar was filled to the shoulder with distilled water. Tap water of high quality was used in cases where no interference could be caused by the dissolved matter.

In the preparation of the samples of fresh plant used in these experiments, the material was passed through a food chopper equipped with the rotary cutter of 1-cm orifice measured at the circumference. The hash was then thoroughly mixed, any juice that had been squeezed out being added, a portion was taken for moisture determination, and the remainder was weighed into sample jars as rapidly as possible.

The specimens preserved at Washington were then stored at definite temperatures until required for use. Since field specimens could be received at this laboratory within 7 days after collection it was accepted, at first, that a preservative that would prevent undue change in the specimen for that length of time would be suitable for use. Consequently most of the early experimental samples were not kept for longer periods. However, when it was found that some substances seemed to possess very good preservative powers a number of experi-

ments were conducted to determine the length of time specimens might be stored and still yield accurate figures on analysis.

METHOD OF ANALYZING SPECIMENS FOR HYDROCYANIC CONTENT

The hydrocyanic acid content of the various samples was determined by the method of Liebig as modified by Denigès (*12*). No difficulty that could be traced to the plant alone was experienced, but on several occasions the preservative rendered the end point obscure or titration quite impossible. The plant specimens were customarily washed into a 5-liter flask with enough water to make 1,200 to 1,500 cc of final volume, and the mixture was distilled over a free flame into 10 cc of 5-percent potassium hydroxide solution. Approximately 400 cc of distillate was taken and titrated, after which distillation was continued until only insignificant quantities of cyanide collected in the receiver.

SOURCES OF ERROR

The principal source of error in this study originated in the method of sampling the green plant. It was impossible to obtain a homogeneous mixture from which several samples might be taken for comparative analyses without allowing considerable loss of hydrocyanic acid and so rendering the results worthless. Since the distribution of cyanogenetic substances is not uniform in various types of tissues in the plant, any excess of one type will alter the hydrocyanic acid content of the sample. Even in samples restricted to leaves there is a difference in the various portions of the leaf structure (*25*), and with samples that contain a portion of stalk, which is fibrous and difficult to comminute and distribute uniformly through the ground sample, the probability of error is much increased. Dried material may be ground to fine powder and intimately mixed so that error from this source is much diminished but not completely eliminated.

Another source of possible error arose in the transference of the stored sample to the flask in which it was to be distilled. With warm samples from the incubator at 37° C., especially when the hydrocyanic acid content is high, there is a possibility that hydrocyanic acid vapor may escape while the sample is being transferred. In cases in which this loss was likely to be appreciable, the samples were weighed into the distilling flask and the preservative solution was added at 37° if the sample was to be incubated. The flask was then tightly stoppered and was not opened until it was connected to the condenser for the actual distillation.

The probable loss of hydrocyanic acid by reaction in the contents of the flask during distillation appears to be a source of error inherent in the process. Aside from losses of hydrocyanic acid due to incomplete cyanogenesis and disappearance of formed hydrocyanic acid by processes in the stored sample, the error inherent in the method may reach 1 percent. Since precision of the order attained in ordinary inorganic analytical procedure is not required in studies of the type reported in this paper, an error of this magnitude is small as compared with experimental differences and does not invalidate the conclusions to be drawn from the experiments. Much more serious are the errors derived from incomplete cyanogenesis and from loss of hydrocyanic acid by apparent conversion into other substances.

PRESERVATIVE ACTION OF DIFFERENT SUBSTANCES

Since the absolute hydrocyanic acid content of the plants under investigation was not known and the figures obtained by various experimental treatments differed within wide limits, it was necessary arbitrarily to select one of the results as a standard with which the other figures and processes might be compared. Ordinarily the highest figure obtained was selected as the standard. It represents the figure that the chemist would report as the hydrocyanic acid content of the sample.

It was found convenient to calculate the analytical results in milligrams of hydrocyanic acid per 100 g of plant rather than in percent. Consequently all the tabular data are expressed in that unit. Figures for green plant are reported on a green rather than on a dry basis since no moisture determinations are available for plants collected at a distance and shipped in preservatives.

UNTREATED SAMPLES

Specimens of the plants were ground through a food chopper, and the weighed quantity was placed in the preserving jar. The specimens were then stored at some definite temperature for the stated time and analyzed. The results (table 1) showed that hydrocyanic acid disappeared during storage in larger than permissible quantities when the specimens were stored at temperatures likely to be encountered during shipment from the place of collection to the laboratory. As indicated by table 1, hegari kept at a low temperature suffered no loss in 4 days.

TABLE 1.—*Hydrocyanic acid recovered from fresh plants after storage without added preservative for different periods*

Plant	Period of preservation	Temperature of preservation	Hydrocyanic acid per 100 g of plant in—		Hydrocyanic acid recovered
			Standard	Test specimen	
	Days	*°C.*	*Milligrams*	*Milligrams*	*Percent*
Arrowgrass	6	(1)	163.8	136.8	83.5
Hegari	1	37	16.5	10.5	63.6
Do	4	9	7.56	7.56	100
Prunus serotina	3	25	131.8	114.4	86.8
Do	6	25	131.8	109	82.7
Sudan grass	3	25	116.6	19.4	16.6

[1] Temperature was variable owing to the transport of the samples across country in summer weather.

REFRIGERATION

The observation that hegari apparently did not change after storage for 4 days at 9° C. suggested further study of this means of preservation. A collection of fresh Spur feterita leaves was minced in a food chopper, and 100-g samples were weighted into 5-liter flasks. To one of these, 1,200 cc of water was added and the hydrocyanic acid was distilled. The sample gave 14 mg of hydrocyanic acid per 100 g of plant. A second sample was mixed with 1,200 cc of water, incubated 24 hours at 37°, and then gave 26.5 mg of hydrocyanic acid per 100 g. This figure was taken as the standard. A third sample

was allowed to remain without the addition of any other substance for 36 hours at laboratory temperature (25°) and then analyzed. It gave 3 mg per 100 g. The other samples were put in two refrigerators one at 9° and the other at 10°. After 24 hours, five of the samples in the refrigerator at 9° were removed, one was mixed with 1,200 cc of water and distilled, and the remaining four were placed in a cold-storage chamber at −10°. The refrigerated samples were withdrawn at 24-hour intervals beginning at 48-hour intervals, mixed with 1,200 cc of water, and the hydrocyanic acid distilled. The results are reported in table 2. None of the refrigerated samples gave as much hydrocyanic acid as the standard, the quantity ranging from 46 to 66.2 percent of the criterion. To determine whether this result was due to suspension of cyanogenesis during refrigeration, one sample was incubated at 37° with 1,200 cc of water for 24 hours. It yielded only 4.05 mg, or 15.3 percent, of the standard. The experiment showed that the mechanism by which hydrocyanic acid is destroyed was considerably increased by the treatment. The results indicated that refrigeration as a method of preservation would not lead to accurate results so far as sorghums are concerned.

TABLE 2.—*Recovery of hydrocyanic acid from fresh Spur feterita leaves after refrigeration under varying conditions*

Period of preservation and temperature (° C.)	Hydrocyanic acid in 100 g of test specimen	Hydrocyanic acid recovery	Period of preservation and temperature (° C.)	Hydrocyanic acid in 100 g of test specimen	Hydrocyanic acid recovery
	Milligrams	*Percent*		*Milligrams*	*Percent*
None	14	52.8	24 hours at 9° then 48 hours at −10°; 24 hours at 37°	4.05	15.3
24 hours at 37° [1]	26.5	100.0	24 hours at 9° then 72 hours at −10°	15.7	59.2
36 hours at 25°	3	11.3	24 hours at 9° then 96 hours at −10°	15.77	59.5
24 hours at 9°	15.5	58.5			
24 hours at 10°	12.2	46.0			
48 hours at 9°	15.5	58.5			
96 hours at 9°	17.55	66.2			
120 hours at 9°	12.69	47.9			
24 hours at 9° then 48 hours at −10°	15.5	58.5			

[1] Taken as the standard.

WATER ALONE

A few specimens were stored with water as the only added substance, with the results shown in table 3.

TABLE 3.—*Hydrocyanic acid recovered from fresh plants after storage in water for 3 or 6 days*

Plant	Period of preservation	Temperature of preservation	Hydrocyanic acid per 100 g of plant in—		Hydrocyanic acid recovered
			Standard	Test specimen	
	Days	*° C.*	*Milligrams*	*Milligrams*	*Percent*
Arrowgrass	6	(1)	163.8	163.8	100
Prunus serotina	3	25	131.8	123	93.3
Sudan grass	3	25	116.6	96.2	82.5

[1] See footnote 1, table 1.

CHLOROFORM MIXTURES

A number of experiments were made with chloroform alone and mixed with water or alcoholic potassium hydroxide (3 percent), or both. In one experiment the alkali was replaced by tartaric acid, which showed a high degree of preservation. In this test 100 g of ground hegari was mixed with 300 cc of water containing 5 g of tartaric acid and 2 cc of chloroform, incubated 24 hours at 37°, and then stored for 17 hours at 9°. The mixture gave 7.38 mg of hydrocyanic acid per 100 g of plant as against 7.56 mg in the standard, or a recovery of 97.6 percent. In one experiment, as shown in table 4, a larger yield of hydrocyanic acid was obtained after 4 days of preservation of hegari with chloroform alone than after 1 day of incubation with water alone, the figure for which is taken as standard. This result may be explained by assuming that chloroform depressed the hydrolysis of the hydrocyanic acid during the time of storage. On the whole the mixtures used cannot be considered satisfactory preservatives, and in addition the presence of chloroform in the distillates during analysis caused a milkiness at times that interfered with observation of the end point.

TABLE 4.—*Hydrocyanic acid recovered from fresh plants after storage with chloroform mixtures for different periods*

Composition of preservative			Plant	Period of preservation	Temperature of preservation	Hydrocyanic acid per 100 g of plant in—		Hydrocyanic acid recovered
Chloroform	Alcoholic potassium hydroxide	Water				Standard	Test specimen	
Cc	*Cc*	*Cc*		*Days*	*°C.*	*Milligrams*	*Milligrams*	*Percent*
2			Arrowgrass	6	(1)	163.8	139.9	85.4
2		300	do	6	(1)	163.8	146	89.1
2			Hegari	4	25	7.56	8.2	108.4
2		300	do	1	9	17.75	17.8	100.0
4			do	1	37	10	9.2	92.0
2	20		do	4	25	7.56	6	79.4
2	20		do	8	25	7.56	6	79.4
2	20	280	*Prunus serotina*	1	25	72.4	23.8	32.9
4			do	1	37	58.9	54.0	91.7
2			do	3	25	131.8	113.4	86.0
2	20	280	Spur feterita	7	25	30.4	10.4	34.2
2		300	Sudan grass	3	25	116.6	96.2	82.5
2		300	White Italian broomcorn	30	25	26.5	21.6	81.5
2		300	do	60	25	26.5	16.2	61.1

[1] See footnote 1, table 1.

ACIDS

Although it is well known that increasing the hydrogen-ion concentration of cyanogenetic mixtures results in a decreased yield of hydrocyanic acid (*11*, *21*, *27*, *29*), it was considered advisable to obtain data on this phenomenon under the conditions of these experiments. Various strengths of different acids in water were added to samples of the plants, and some of the mixtures were then incubated for 24 hours at 37° C. Several of these were then stored in an ice box at 9° for a further 17 hours before analysis.

TABLE 5.—*Hydrocyanic acid recovered from fresh plants after storage with different acids in the dilution indicated for different periods*

Acid	Strength of preservative[1]	Water, as diluent	Plant	Period of preservation	Temperature of preservation	Hydrocyanic acid per 100 g of plant in—		Hydrocyanic acid recovered
						Standard	Test specimen	
	Per cent	*Cc*		*Hours*	*°C.*	*Milligrams*	*Milligrams*	*Per cent*
Aluminum sulphate [2]	6	1,000	*Prunus serotina*	1	25	116.3	95.2	81.9
Lactic	3	300	Sudan grass	24	37	16.5	15.4	93.3
Do	1	300	do	24	37	16.5	12.7	76.9
Phosphoric	4	300	do	41	[3]	7.56	3.88	51.3
				Days				
Salicylic	1	300	White Italian broomcorn	3	25	26.5	24.8	93.6
Do	1	300	Sudan grass	3	25	116.6	109	93.5
Do	1	300	*P. serotina*	3	25	131.8	127.4	96.7
Do	1	300	do	3	25	131.8	125.3	95.0
Do	5	300	Arrowgrass	6	[4]	163.8	147.7	90.2
				Hours				
Sulphuric	2	300	Sudan grass	41	[3]	7.56	5.68	75.1
Do	3	300	do	24	37	16.5	3.5	21.2
Do	5	300	White Italian broomcorn	1	25	26.35	5.4	20.5
Tartaric	10	300	Hegari	41	[3]	7.56	6.59	87.1
Do	10	300	do.[5]	41	[3]	7.56	6.76	89.4
Do	5	1,000	do.[5]	41	[3]	10	10.8	108.0
Do	5	300	do	24	37	16.5	12.2	73.9

[1] Calculated on weight of plant.
[2] Acid in reaction, though not technically an acid.
[3] 24 hours at 37° and 17 hours at 9°.
[4] See footnote 1, table 1.
[5] Frosted.

The results are reported in table 5, the strength of the acid being calculated to the weight of plant and not to the volume of liquid. Thus, 10 percent of acid refers to 10 g of acid per 100 g of plant used in the experiment. The results obtained from the use of the different acids were in general similar, the differences noted suggesting that the effective agent in loss of hydrocyanic acid is the concentration of hydrogen ions. The greatest effects were obtained with sulphuric and phosphoric acids and less with weak acids, such as lactic and salicylic. Indeed, if salicylic acid did not distill with the hydrocyanic acid and tend to interfere with the subsequent titration it would merit further consideration as a preservative. The effect of lactic acid was of interest because of its possible formation in silage and the findings of Collins (*11*) that lactic acid markedly depresses the rate of evolution of hydrocyanic acid from linseed. The question whether the observed effects of various acids are to be referred solely to hydrogen-ion concentration or whether the character of the anion is also a factor is being investigated in this laboratory.

ALKALIES

A few experiments were made to determine the effect of alkalies on the cyanogenetic plants used in this study. These tests were usually made in connection with experiments on other preservatives and served to furnish comparative data. The results obtained, reported in table 6, are in agreement with those found by Swanson, (*23*) Auld, (*5*) and others. They raise the important question whether the effect of the alkali is inhibition of the cyanogenesis or destruction of the

hydrocyanic acid after its formation. The data, although too few in number to permit conclusions, indicate the probability of the latter alternative. With *Prunus serotina*, in which ordinarily cyanogenesis takes place very rapidly, there was an 80 to 90 percent decrease in hydrocyanic acid recovered, whereas with Sudan grass, in which cyanogenesis is somewhat slower, the decrease was 91 to 96 percent. With alcoholic potassium hydroxide, in which case the alcohol would be expected to repress cyanogenesis and so act to heighten the effect of the alkali if the first alternative were true, actually the contrary effect was noted. Increasing the quantity of alcohol present resulted in a larger yield of hydrocyanic acid.

TABLE 6.—*Hydrocyanic acid recovered from fresh plants after storage in alkaline liquids in the dilutions indicated*

Preservative	Strength of preservative	Diluent [1]	Plant	Period of preservation	Temperature of preservation	Hydrocyanic acid per 100 g of plant in—		Hydrocyanic acid recovered
						Standard	Test specimen	
	Pct.			*Days*	*°C.*	*Milligrams*	*Milligrams*	*Pct.*
Calcium hydroxide	2	Water	Sudan grass	3	25	116.6	10	8.6
Do	5	do	*Prunus serotina*	3	25	131.8	27.0	20 5
Do	10	do	do	3	25	131.8	21.6	16.4
Magnesium oxide	5	do	Arrowgrass	6	(2)	163.8	57.8	35.3
Potassium hydroxide	2	25 per cent alcohol	*P. serotina*	3	25	158.8	14	8.8
Do	2	50 percent alcohol	do	3	25	158.8	21.6	13.6
Sodium carbonate	2	Water	White Italian broomcorn.	3	25	35.6	11.8	33.2
Do	2	do	Sudan grass	3	25	116.6	4.4	3.8
Do	2	do	*P. serotina*	3	22	158.8	14.6	9.2
Do	2	do	Arrowgrass	6	(2)	163.8	112.1	68.4

[1] 300 cc was used in all cases.
[2] See footnote 1, table 1.

MISCELLANEOUS PRESERVATIVES

The results obtained in tests of a number of substances which proved not to be suitable are reported in table 7. Toluene was used because of its well-known antiseptic action. With one sample of hegari it yielded an increased quantity of hydrocyanic acid. However, during the experiment, the mixture was stored at a low temperature, 9° C., a condition under which another sample of hegari without preservative lost no hydrocyanic acid during 4 days' storage (table 1). With samples of other plants the loss of hydrocyanic acid was too large to warrant the further use of toluene which, in addition, has the disadvantage of distilling over with the hydrocyanic acid and interfering somewhat with the titration.

Glucose, thymol, and hexone added to samples of *Prunus serotina* resulted in mixtures that yielded as much hydrocyanic acid after 24 hours as did a sample incubated at the same temperature for 24 hours with the addition of water only. Thymol and hexone distill with the hydrocyanic acid and may interfere with the titration.

TABLE 7.—*Hydrocyanic acid recovered from fresh plants after storage with miscellaneous preservatives in water solution for 1 to 3 days*

Preservative	Strength of preservative	Plant	Period of preservation	Temperature of preservation	Hydrocyanic acid per 100 g of plant in—		Hydrocyanic acid recovered
					Standard	Test specimen	
	Percent		*Days*	*°C.*	*Milligrams*	*Milligrams*	*Percent*
Toluene (alone)	2	*Prunus serotina*	3	25	131.8	113.4	86.0
Do	2	do	3	25	131.8	100.0	79.5
Do	2	White Italian broomcorn	1	25	26.5	24.3	91.7
Do	4	Hegari	1	9	17.75	19.4	109.3
Glucose	4	*P. serotina*	1	37	72.4	72.4	100.0
Thymol	4	do	1	37	72.4	73.4	101.4
Hexone	8	do	1	37	72.4	72.4	100.0
Aniline	2	White Italian broomcorn	3	25	26.5	23.8	89.8
Do	4	*P. serotina*	3	25	161	161	100.0
p-Toluidine	4	do	3	25	161	149	92.5
Pyridine	4	do	3	25	161	144.8	89.9
Formaldehyde	4	Hegari	1	37	7.56	[1]	[1]
Do	1	do	1	37	7.56	1.76	23.3
Hexamine	1	do	2	9	32.1	9.7	30.2
Do	1	Spur feterita	2	9	22	8	36.4
Tyrosine	0.5	Hegari	[2]	[2]	7.56	7.02	92.9

[1] Not titratable.
[2] 24 hours at 37° followed by 17 hours at 9°.

It was thought worth while to determine whether very weak alkalies such as the organic bases—aniline, *p*-toluidine, and pyridine—might be effective in preventing the loss of hydrocyanic acid without interfering with cyanogenesis. It was found that these substances were effective as compared with inorganic alkalies and might be worthy of further investigation. All of them have the disadvantage that they distill with the hydrocyanic acid and may interfere in the titration.

Formaldehyde, hexamine, and tyrosine were employed in some tests to determine whether the addition of these substances might not increase the quantity of hydrocyanic acid by actual phytosnythesis. Except in the test with tyrosine there was a great decrease of hydrocyanic acid, and in the experiment in which 4 percent of formaldehyde was used so much of the aldehyde was present in the distillate that the reagent was immediately reduced to colloidal silver and no titration could be made. In spite of the low temperature at which the hexamine mixtures were stored there was a considerable loss of hydrocyanic acid, indicating a very direct effect of hexamine on cyanogenesis.

ALCOHOL IN VARIOUS DILUTIONS

The well-known fact that ethyl alcohol in the higher concentrations inhibits the action of enzymes raised a question whether the concentration effective for preserving cyanogenetic plants would not also inhibit the cyanogenesis to such an extent that this feature could not be overcome by subsequent handling of the samples. A series of experiments was conducted to determine (1) whether alcohol possesses sufficient preservative action to be useful, (2) in how low a concentration alcohol might still be effective, (3) how long the preservative action continues, and (4) whether these properties differ with different species of plants. The alcohol used was U. S. P. grade ethanol, which was diluted with distilled water to the desired concentration. The samples were weighed into the standard jars, covered with 300 cc of

the diluted alcohol, and set aside at 25° C. for the time indicated. Three samples of fresh arrowgrass were preserved at Salina, Utah, and shipped by express to the laboratory and so were exposed to various temperatures incidental to transport across the country during August. The data, reported in table 8, show that alcohol is a good preservative for short periods in low concentrations and for the four species represented by the plants studied. Table 9 contains a rearrangement of certain of these data to show the effect of different concentrations of alcohol with different species. In concentrations of 25 percent and lower alcohol gave good results. The higher concentrations used indicated some decrease of the cyanogenesis presumably by inhibition of enzyme action.

TABLE 8.—*Hydrocyanic acid recovered from fresh plants after storage with varying percentages of alcohol for different periods*

Alcohol (percent)	Plant	Period of preservation	Temperature of preservation	Hydrocyanic acid per 100 g of plant in—		Hydrocyanic acid recovered
				Standard	Test specimen	
		Days	*°C.*	*Milligrams*	*Milligrams*	*Percent*
10	Sudan grass	3	25	116.6	114.5	98.2
	Do	6	25	116.6	114.5	98.2
	Prunus serotina	3	25	161	168.5	104.6
	White Italian broomcorn	4	25	35.6	33.5	94.1
	Do	3	25	26.5	24.8	93.6
15	Sudan grass	3	25	116.6	116.6	100.0
	P. serotina	3	25	161	175	108.7
	Do.[1]	7	25	116.6	108.6	93.1
	Arrowgrass	6	(2)	163.8	153.4	93.6
	Hegari	14	25	19.7	21.3	108.1
	Do.[1]	21	25	10	9.2	92.0
	Spur feterita	7	25	26.5	28.6	107.9
20	Sudan grass	3	25	116.6	115.6	99.1
	Do	6	25	116.6	113.4	97.2
	Spur feterita	7	25	26.5	27.6	104.1
	Do	7	25	26.5	28	105.7
25	Sudan grass	3	25	116.6	108	92.6
	P. serotina	3	25	131.8	131.8	100.0
	Do	3	25	131.8	140.4	100.6
50	White Italian broomcorn	3	25	26.5	25.4	95.8
	Sudan grass	3	25	116.6	98.2	84.2
	Arrowgrass	6	(2)	163.8	142.8	87.2
	Do	6	(2)	163.8	144.7	88.3
95	White Italian broomcorn	3	25	26.5	14	52.8
	P. serotina	3	25	131.8	92	69.8

[1] Frosted. [2] See footnote 1, table 1.

TABLE 9.—*Hydrocyanic acid recovered from fresh plants after storage in alcohol of varying strength for 3 to 7 days*

Alcohol (percent)	Hydrocyanic acid recovered from—				
	White Italian broomcorn	Spur feterita	Sudan grass	*Prunus serotina*	Arrowgrass
	Percent	*Percent*	*Percent*	*Percent*	*Percent*
10			98.2 98.2	104.6	
15	94.1 93.6	107.9	100.0	108.7 [1] 93.1	93.6
20		104.1	99.1 97.2		
25		105.7	92.6	100.0	
50	95.8		84.2	100.6	87.2
95	52.8			69.8	88.3

[1] Frosted.

The question of the length of time that alcohol might preserve these plants was studied with reference to the three most suitable concentrations—15, 20, and 25 percent. Although 10 percent alcohol had given good results for short periods it was discarded after one trial because of the fact that the moisture in the plant sample, approximately 80 percent on the average, would seriously dilute 10-percent alcohol and the probability is that such a strength would not preserve against bacterial decomposition. Comparison of the effects of 15-, 20-, and 25-percent concentrations on fresh leaves of Spur feterita for 10 weeks are shown in table 10. At the end of the first week there was a small increase in the quantity of hydrocyanic acid obtained as compared with the standard, which was obtained after 24 hours of incubation of the fresh plant in water at 37° C. The rise indicates that cyanogenesis continued for several days and that the 24-hour figure did not represent the true value for this sample. By the end of the second week the yield of hydrocyanic acid began to decrease and from then on there was a steady loss of hydrocyanic acid until the seventh or eighth week, when an equilibrium appeared to have been established after a loss of approximately 20 percent of the original hydrocyanic acid. Similar comparisons were made with white Italian broomcorn and *Prunus serotina*, the results of which are reported in tables 11 and 12.

TABLE 10.—*Hydrocyanic acid recovered from fresh leaves of spur feterita after storage in alcohol of various strengths for different periods*

[Standard: 26.5 mg of hydrocyanic acid per 100 g of plant]

Period of preservation (days)	Hydrocyanic acid per 100 g of plant preserved in—			Period of preservation (days)	Hydrocyanic acid per 100 g of plant preserved in—		
	15-percent alcohol	20-percent alcohol	25-percent alcohol		15-percent alcohol	20-percent alcohol	25-percent alcohol
	Milligrams	*Milligrams*	*Milligrams*		*Milligrams*	*Milligrams*	*Milligrams*
7	28.6	27.6	28.0	42	22.7	22.7	23.3
14	26	26	27	56	21.6	21.1	21.6
21	24.8	25.4	26	70	22.2	22.2	21.1
28	24.4	24.4	24.4				

TABLE 11.—*Hydrocyanic acid recovered from white Italian broomcorn after storage in 15- and 25-percent alcohols for different periods*

[Standard: 26.5 mg of hydrocyanic acid per 100 g of plant]

Period of preservation (days)	Hydrocyanic acid per 100 g of plant preserved in—		Period of preservation (days)	Hydrocyanic acid per 100 g of plant preserved in—		Period of preservation (days)	Hydrocyanic acid per 100 g of plant preserved in—	
	15-percent alcohol	25-percent alcohol		15-percent alcohol	25-percent alcohol		15-percent alcohol	25-percent alcohol
	Milligrams	*Milligrams*		*Milligrams*	*Milligrams*		*Milligrams*	*Milligrams*
10	22.6		45	18.4		87	16.7	14
17	19.4		52	18.4		101	16.2	
24	19.4		59	18.4	17.3	115	14.3	13.2
31	20	21.2	73	17.8		143	15.9	14
38	17.8							

TABLE 12.—*Hydrocyanic acid recovered from Prunus serotina after storage in alcohol of different strengths for different periods*

[Standard: 161 mg of hydrocyanic acid per 100 g of plant]

Period of preservation (days)	Hydrocyanic acid per 100 g of plant preserved in—			Period of preservation (days)	Hydrocyanic acid per 100 g of plant preserved in—		
	10-percent alcohol	15-percent alcohol	20-percent alcohol		10-percent alcohol	15-percent alcohol	20-percent alcohol
	Milligrams	*Milligrams*	*Milligrams*		*Milligrams*	*Milligrams*	*Milligrams*
3	168.5	175	171.7	13	165.2	171.7	169.6
6	166.8	172.8	170.6	20	155.5	162	163.1

From these experiments it appeared that the use of 20- and 25-percent alcohols offered no particular advantage over 15-percent, and this strength was adopted for short-time preservation of cyanogenetic plants. A series of experiments made with fresh Sudan grass and continued for 15 weeks is reported in the following tabulation. Here there was a continuous drop for 2 weeks, when the rate of decrease diminished and equilibrium was established after 7 weeks. At that time the samples had lost approximately 60 percent of their original hydrocyanic acid.

Period of preservation (days)	Hydrocyanic acid per 100 g of plant (milligrams)	Period of preservation (days)	Hydrocyanic acid per 100 g of plant (milligrams)
0	23.1	49	8.6
7	16.2	63	8.6
14	9.8	77	8.1
21	9.8	91	8.1
28	10.2	105	7.7
35	9.2		

It should be noted here that the sample had been collected in Colorado and mailed fresh to the laboratory, where it arrived in good condition, free from mold or decomposition but undoubtedly altered in certain respects.

A sample of hegari which yielded 19.7 mg of hydrocyanic acid per 100 g after 24 hours of incubation in water was preserved in 15-percent alcohol. After 2, 11, and 19 weeks specimens yielded 21.3, 13.3, and 13.8 mg of hydrocyanic acid per 100 g, respectively, or recoveries of 108.1, 67.5, and 70.1 percent.

From the data presented it appears that although 15-percent alcohol preserves samples for 7 to 10 days without serious loss, it cannot be depended on for longer periods. However, data subsequently obtained show clearly that the figures for the hydrocyanic acid content of plants, obtained after incubation, even for as long as 24 hours, are not necessarily accurate. Data obtained by the use of 15-percent alcohol have a certain value for purposes of comparison but more than this cannot be expected of them.

MERCURIC CHLORIDE

It appeared very early in this investigation that if use could be made of the property of mercuric chloride by which it reacts with

cyanides to form nonionized mercuric cyanide, which is one of the most stable of all cyanides, loss of hydrocyanic acid in preserved samples could be prevented for long periods and a solution of the problem reached. Consideration of the use of this substance, however, brought up a number of questions. Aside from the uncertainty whether mercuric cyanide would be stable in a mixture of plant constituents, the nature and action of many of which are quite unknown, there arose the problem (1) whether mercuric chloride might not inactivate the enzymes of the plant and so prevent cyanogenesis, and (2) the quantitative recovery of hydrocyanic acid from the mixture after storage. Since mercuric cyanide is not very reactive and hydrocyanic acid is very labille, the choice of reagents and technique available to liberate the acid was narrowly limited.

Preservative Properties of Mercuric Chloride

Experiments were made to determine whether mercuric chloride actually did have any preservative properties in these plant mixtures. The general results are reported in table 13. Fresh plants were used when available, and except in two instances, 2 percent of the preservative was added in water solution and the sample was customarily diluted to 400 cc with water. It was stored at the indicated temperature for various periods and then analyzed. The standard chosen for comparison was determined as previously described and does not, of course, represent the absolute value for the hydrocyanic acid content of the specimen. With fresh plants those preserved with mercuric chloride showed a distinct increase in hydrocyanic acid recovered. Dried plants, however, after short periods of storage generally yielded smaller quantities of hydrocyanic acid, but on longer storage the quantity increased. It appeared that this effect was due to inhibition of the enzyme either by actual precipitation by the mercuric chloride or possibly by the increased hydrogen-ion concentration resulting from the hydrochloric acid liberated during the formation of mercuric cyanide. To test the latter possibility several experiments were made in which the mercuric chloride was treated with a molecular proportion of sodium citrate before addition to the plant. These buffered mixtures did not regularly increase the yield of hydrocyanic acid sufficiently to indicate that the hydrogen-ion concentration is the important factor in the inhibition. Preliminary experiments in which enzyme was added, however, showed a definite and rapid response and further study of this process was indicated. It was thought possible that some method of accelerating the enzyme action might solve the problem. Guignard (*15*) and Mirande (*16*) have reported that cyanogenetic plants treated with chloroform and other anesthetics evolve hydrocyanic acid more rapidly than untreated plants. This appeared to be due to a possible acceleration of cyanogenesis. Accordingly chloroform was added to two samples of dried Johnson grass preserved with 2-percent mercuric chloride, and the mixtures were stored for 1 and 2 says at 37° C. The results indicated a retarding effect of the chloroform. The effects reported by Guignard and Mirande therefore appear to be due to some other mechanism.

TABLE 13.—*Hydrocyanic acid recovered from plants with mercuric chloride solutions for different periods*

Mercuric chloride (percent)	Plant	Condition of plant	Period of preservation	Temperature of preservation	Hydrocyanic acid per 100 g of plant in—		Hydrocyanic acid recovered
					Standard	Test specimen	
			Days	*° C.*	*Milligrams*	*Milligrams*	*Percent*
2	Arrow grass	Dried	3	37	400.1	387	96.7
2	do	do	1	37	158.8	128	80.6
2	Hegari	Fresh	77	25	19.7	20.3	103.0
2[1]	do	do	77	25	19.7	22	111.7
2	do	do	133	25	19.7	22	111.7
2[1]	do	do	133	25	19.7	22.2	112.7
2	Johnson grass	Dried	13	37	294	283	96.3
			Hours				
2[2]	do	do	4	37	294	184.7	62.8
			Days				
2[2]	do	do	2	37	294	313	106.5
2[3]	do	do	1	37	294	134.6	45.9
2[3]	do	do	2	37	294	168.6	57.3
2	*Prunus melanocarpa*	do	1	37	215.3	260.8	121.1
2	do	do	62	25	178.1	196.6	110.4
2	*P. serotina*	Fresh	112	25	161	178.9	111.1
2	do	do	7	25	116.6	126.4	108.4
4	do	do	7	25	116.6	122.0	104.6
10	do	do	28	25	116.6	124.2	106.5
2	Spur feterita	Fresh	4	37	30.4	33.4	109.9
2[1]	do	do	3	37	30.4	33.8	111.2
2[1]	do	do	3	37	30.4	32.4	106.6
2[1]	do	do	4	37	30.4	32.8	107.9

[1] Treated with a molecular proportion of sodium citrate before addition to the plant.
[2] Enzyme added.
[3] 2 cc of chloroform added.

INHIBITION OF CYANOGENESIS BY MERCURIC CHLORIDE

A study of the comparative preservative efficiency of 15-percent alcohol and 2- and 4-percent mercuric chloride solutions furnished evidence of the inhibitory effect of the salt. Samples of a single collection of fresh *Prunus serotina* collected near the laboratory and passed through a food chopper were preserved in the three solutions and analyzed at intervals for 22 weeks. The results are reported in table 14 and plotted in figure 1.

TABLE 14.—*Hydrocyanic acid recovered from fresh Prunus serotina leaves after storage in 15 percent alcohol and 2- and 4-percent mercuric chloride solutions for 1 to 22 weeks*

Period of preservation (weeks)	Hydrocyanic acid per 100 g of plant in—			Period of perservation (weeks)	Hydrocyanic acid per 100 g of plant in—		
	15-percent alcohol	2-percent mercuric chloride	4-percent murcuric chloride		15-percent alcohol	2-percent mercuric chloride	4-percent mercuric chloride
	Milligrams	*Milligrams*	*Milligrams*		*Milligrams*	*Milligrams*	*Milligrams*
1	108.6	126.4	122	8		126.5	
2	106.9	124.2	119.9	10	94.4	127.7	123.9
4		125.2		15		132.1	128.4
6	100.4	126.4	121.5	22	88.7	133.2	

The curves clearly show the excellence of the preservative action of the mercuric chloride as compared with 15-percent alcohol, and the inhibiting effect is indicated by the lower values obtained with 4-percent as compared with 2-percent mercuric chloride. The fact that in both cases there is a steady increase in hydrocyanic acid over the whole period after the first week indicates that the action of the bichloride is an inhibition rather than a destruction of the enzyme.

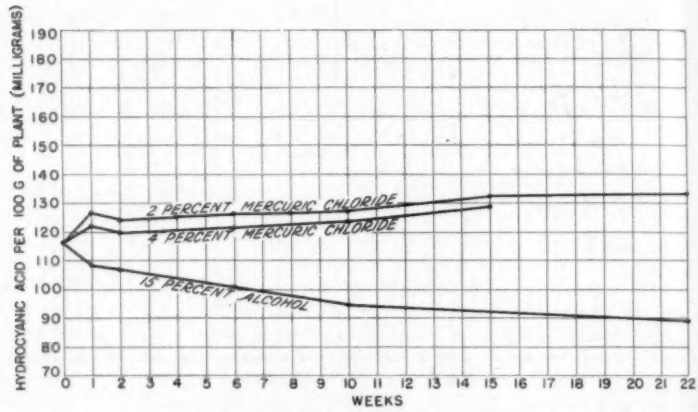

FIGURE 1.—Hydrocyanic acid recovered from fresh cherry leaves (*Prunus serotina*) preserved in 15-percent alcohol and 2- and 4-percent mercuric chloride solution.

A similar effect was observed in a series of experiments in which dried Johnson grass was used. Samples weighing 25 g were mixed with water and with a 2-percent mercuric chloride solution, and to a few samples of each enzyme was added. All were stored at 37° C. and analyzed at intervals. The samples that were mixed with water only were withdrawn at 2-hour intervals during the first day and analyzed for hydrocyanic acid content; the rest of these samples and samples that were mixed with mercuric chloride solution were withdrawn at longer intervals. The results are reported in table 15. Here the inhibitory effect of the bichloride is evident. When the plant was incubated with water alone the highest yield of hydrocyanic acid was obtained on the sixth day, whereas in the case of the bichloride the yield of hydrocyanic acid after 6 days was only 90.7 percent of that in water and the comparable yield was not obtained until the thirteenth day. At the end of 24 hours, however, samples to which enzyme had been added yielded larger quantities of hydrocyanic acid than either of the other series, and at the end of 48 hours the sample preserved in mercuric chloride to which enzyme had been added yielded the largest quantity of hycrocyanic acid obtained in the entire experiment.

The data for the samples mixed with water alone disclose a phenomenon, observed regularly in studies of this kind, which consists in a decrease in quantity of hydrocyanic beginning after some 10 hours of incubation and continuing for a variable period of 6 to 12 hours, when

the yield again reaches the former level and continues to increase. This drop has been consistently noted with several cyanogenetic plants, and it is planned to make it the subject of investigation to determine the factors which are operating.

TABLE 15.—*Rate of formation of hydrocyanic acid in dried Johnson grass stored in water and in 2-percent mercuric chloride solution at 37° C. for different periods*

Period of preservation	Hydrocyanic acid per 100 g of plant preserved in—				Period of preservation	Hydrocyanic acid per 100 g of plant preserved in—			
	Water	Mercuric chloride	Mercuric chloride and enzyme	Water and enzyme		Water	Mercuric chloride	Mercuric chloride and enzyme	Water and enzyme
Hours	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Hours*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*
0	123.1				42	214.9			
2	136.6				*Days*				
4	139.8		143.6	186	2	222.4	189.7	313	
6	150.5	101.3			3	262	211.7		
8	156.6				4	267.5	232.3		
10	196.1				5	273.8	258.1		
12	181.4				6	283.7	257.4		
14	185.4				7	278.6	265.9		
16	184				8		267		
18	186.4	136.1			9		271.6		
20	188.4				10		272.9		
22	198.3				11		278.2		
24	205.1	154	294.1	294.4	12		278.9		
30	210.4				13		283		
36	220.3	169.6							

The inhibiting effect of mercuric chloride is also shown in experiments with dried arrowgrass in which results obtained with storage of plants in water are compared with results from storage in 2-percent mercuric chloride. The data are reported in table 16. They show a retardation in the formation of hydrocyanic acid at periods between the sixth and eighteenth hours. The samples preserved in mercuric chloride did not reach quite as high a yield as those preserved with water alone at the end of the experiment, when the material was exhausted.

TABLE 16.—*Rate of formation of hydrocyanic acid in dried arrowgrass stored in water and 2-percent mercuric chloride solution at 37° C. for different periods*

Period of preservation (hours)	Hydrocyanic acid per 100 g of plant preserved in—		Period of preservation (hours)	Hydrocyanic acid per 100 g of plant preserved in—	
	Water	Mercuric chloride		Water	Mercuric chloride
	Milligrams	*Milligrams*		*Milligrams*	*Milligrams*
0	149.7		16	381.9	
2	376.7		18	393.2	
4	382.1		20	400.1	
6	385.2		22	399	
10	360.5		24	394.4	381.8
12	391	378.3	48	402.4	387.9
14	391		72		387

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Since it was possible to obtain 313 mg of hydrocyanic acid per 100 g of Johnson grass by adding enzyme, whereas the use of water alone gave 283.7 mg as the highest, it is evident that either cyanogenesis was incomplete in the water solution or hydrocyanic acid was lost in some manner. If the latter be true it follows that the figures obtained in the analysis of cyanogenetic plant mixtures with water represent the resultant of two processes: (1) the formation of hydrocyanic acid by cyanogenesis, and (2) the disappearance of hydrocyanic acid either by hydrolysis to ammonium formate or conversion into some other compound. The disappearance of hydrocyanic acid under circumstances in which loss by volatilization is excluded, as in closed systems, was illustrated in a series of determinations made on dried Sumac sorghum, in which weighed samples of plant were mixed with water, stored at 37° C., and withdrawn at 2-hour intervals for 24 hours, omitting the 8-hour determination. The samples were analyzed for hydrocyanic acid content immediately upon withdrawal. The results are reported in the following tabulation:

Period of storage (hours)	Hydrocyanic acid per 100 g of plant (milligrams)	Period of storage (hours)	Hydrocyanic acid per 100 g of plant (milligrams)
0	83.2	14	74.5
2	78.8	16	71.3
4	77.8	18	67.4
6	77.8	20	65.8
10	73.4	22	59.4
12	75.6	24	34.6

The loss of 58.4 percent may be considered to represent only a part of the actual loss of hydrocyanic acid in this case since it is not likely that the first figure, 83.2 mg, includes the total potential hydrocyanic acid present in the plant. The fact that such large losses can occur was confirmed with other specimens of dried sorghums.

That this loss is not always apparent and that data obtained in similar experiments may be misleading were indicated in a series of experiments conducted on a sample of dried (*Prunus melanocarpa*, in which 25-g samples were stored with water and with 2-percent mercuric chloride solutions and analyzed at intervals for 24 hours. The results are reported in table 17 and are plotted in figure 2. The curve for hydrocyanic acid in the samples stored in water over the 24-hour period again exhibits a depression similar to that noted in the case of Johnson grass with a recovery and subsequent drop. The loss of hydrocyanic acid after 24 hours as shown by the data is only 5 percent, much less than was noted with Sumac sorghum. However, the curve for the portion of the sample preserved in mercuric chloride shows that the true value for the hydrocyanic acid content of the sample was at least 260.5 mg. There was, then, a loss of at least 45.2 mg, or 17.4 percent, during the 24-hour period. Such considerations raise the serious question whether any hydrocyanic acid determination is reliable in which account of the possible loss of hydrocyanic acid during analysis has not been taken.

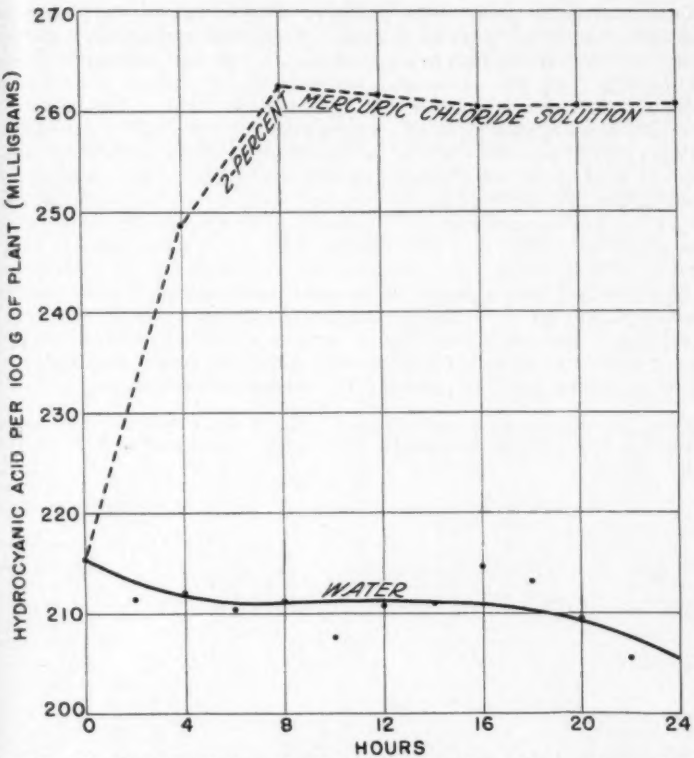

FIGURE 2.—Rate of formation of hydrocyanic acide from dried cherry leaves (*Prunus melanocarpa*) stored in water and in 2-percent mercuric chloride solution.

TABLE 17.—*Rate of formation of hydrocyanic acid in dried Prunus melanocarpa stored in water and in 2-percent mercuric chloride solution for different periods*

Period of storage (hours)	Hydrocyanic acid per 100 g of plant preserved in—		Period of storage (hours)	Hydrocyanic acid per 100 g of plant preserved in—		Period of storage (hours)	Hydrocyanic acid per 100 g of plant preserved in—	
	Water	Mercuric chloride		Water	Mercuric chloride		Water	Mercuric chloride
	Milligrams	*Milligrams*		*Milligrams*	*Milligrams*		*Milligrams*	*Milligrams*
0	215.3		10	207.8		18	213.2	
2	211.6		12	210.9	261.6	20	209.5	260.7
4	212.1	248.6	14	211		22	205.5	
6	210.5		16	214.6	260.5	24	205.1	260.8
8	211.1	262.6						

PERIOD OF EFFECTIVE PRESERVATION BY MERCURIC CHLORIDE

A number of experiments were made to determine how long the hydrocyanic acid content of plants treated with mercuric chloride

solutions may be preserved. Samples of fresh plants were collected, minced in the usual way, thoroughly mixed, and weighed into preservative jars, and immediately mixed with 1 percent of mercuric chloride in solution. At the same time, as controls, untreated samples were selected for determination of hydrocyanic acid. The preserved samples were stored at 25° C. and specimens were taken for analysis after various periods. Data for samples of *Prunus serotina* leaves and of four varieties of sorghum are reported. The standard was determined by macerating the ground sample for 24 hours in water at 37° and then distilling. The results of representative experiments are given in table 18. The figures show good preservation over a period of 6 months. In one instance the quantity of hydrocyanic acid recovered from a preserved sample was less than for the previous period of storage, but this result may have been due to an error of sampling. The uniformly higher figures obtained after 4 weeks of preservation as compared with the standard again emphasize the error in determinations made by the customary technique.

TABLE 18.—*Hydrocyanic acid recovered from Prunus serotina and sorghum varieties after storage in 1-percent solutions of mercuric chloride for different periods*

Plant	Hydrocyanic acid in 100 g of fresh plant in—				Plant	Hydrocyanic acid in 100 g of fresh plant in—			
	Standard	Test specimens stored for—				Standard	Test specimens stored for—		
		4 weeks	12 weeks	6 months			4 weeks	12 weeks	6 months
	Milligrams	*Milligrams*	*Milligrams*	*Milligrams*		*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*
Chiltex	14.6	16.3	.....	16.4	Spur feterita	10.4	11.6	12.5	12.7
Kansas Orange	15.3	15.7	.....	15.8		26.3	32.3	31.3	33.3
Prunus serotina	98.6	111.7	114.0	.....	Sumac	8.0	12.0	12.0	.....
	112.1	121.0	.....	.....					

STRENGTH OF MERCURIC CHLORIDE NECESSARY FOR PRESERVATION

The strength of mercuric chloride needed to insure preservation was the subject of several series of experiments with dried and fresh sorghums. Since preservation was found to exert a retarding effect on cyanogenesis, it is desirable to reduce the strength as much as possible without loss of preservative power. In the experiments different quantities of mercuric chloride were used and the storage periods were extended as long as the specimen material lasted. The results, reported in table 19, indicate that 1 percent, calculated on the weight of plant, is necessary for fresh plants. In the case of dried plants, 2 percent preserved reasonably well for 3 months, but after that time there was a considerable loss. indicating hat a higher percentage is required for dried sorghum. For dried cherry (*Prunus melanocarpa*) 2-percent mercuric chloride preserved satisfactorily for 6 months (table 20).

TEMPERATURE OF STORAGE IN MERCURIC CHLORIDE

Experiments conducted on dried samples of *Prunus melanocarpa* stored in mercuric chloride at 9°, 25°, and 37° C. indicated a small

advantage for those stored at 25°. These results are reported in table 20. A series of samples stored with water at 37° for comparison shows the great loss of hydrocyanic acid that occurs under these conditions.

TABLE 19.—*Hydrocyanic acid recovered from sorghums preserved with mercuric chloride at 37° C. in varying concentrations for different periods*

Plant	Period of preservation	Hydrocyanic acid per 100 g of plant preserved in—					
		Water	Mercuric chloride				
			0.25 percent	0.5 percent	0.75 percent	1 percent	2 percent
		Milligrams	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*
Fresh hegari	0	11.7					
Do	24 hours	11.4					
Do	2 weeks		15.8	15.5	15.6	15.8	
Do	3 weeks		17	18.1	19.4	20.8	
Do	5 weeks		15.5	20.2	18.6	20.4	
Do	7 weeks		13	[1] 24.2	18.4	25.9	
Do	9 weeks			19.4	19.8	22.5	
Dried hegari	0	32					
Do	24 hours	31.8					
Do	4 weeks			54.8		61.8	69.7
Do	3 months			44.4		47.9	67.6
Do	5 months			30.6		34.9	55.3
Do.[2]	0	30.7					
Do	24 hours	37.1					
Do	1 week			63.8		70.2	69.4
Do	2 weeks			61.2		66.8	69.9
Do	3 weeks			55.6		67.3	70.1
Do	4 weeks			54.3		64.2	70.2
Dried Chiltex	0	51.3					
Do	24 hours	66.2					
Do	4 weeks						82.6
Do	3 months						81.2
Do	5 months						56.3
Do.[3]	0	54.1					
Do	24 hours	73.4					
Do	1 week						91.75
Do	2 weeks						92.7
Do	3 weeks						93.2
Do	4 weeks						95

[1] End point obscured by darkening.
[2] Whole sample, finely ground.
[3] Finely ground; coarse fibers discarded.

TABLE 20.—*Hydrocyanic acid recovered from dried Prunus melanocarpa after storage in 2-percent mercuric chloride solution at various temperatures for different periods*

Period of preservation	Hydrocyanic acid per 100 g of plant preserved in—				Period of preservation	Hydrocyanic acid per 100 g of plant preserved in—			
	Water at 37° C.	2-percent mercuric chloride at—				Water at 37° C.	2-percent mercuric chloride at—		
		9° C.	25° C.	37° C.			9° C.	25° C.	37° C.
	Milligrams	*Milligrams*	*Milligrams*	*Milligrams*		*Milligrams*	*Milligrams*	*Milligrams*	*Milligrams*
0	162.5				4 months	84.9	196.4	201.8	196.3
24 hours	178.1				6 months	71.5	199	201.4	190.2
2 months		194.4	197.6	196.6	8 months	61.7		197.2	

Recovery of Hydrocyanic Acid From Mercuric Cyanide

One of the most important problems connected with the mercuric chloride method of preservation was the quantitative recovery of hydrocyanic acid from the preserved samples. Two procedures have been reported and both were studied in the present investigation. Rupp and Goy (*22*) suggested the use of potassium iodide to convert mercuric cyanide into mercuric iodide and hydrocyanic acid, and Roe (*19*) advocated the use of stannous chloride, which converts mercuric cyanide into calomel and hydrocyanic acid. Both reactions take place in slightly acid solution, the normal condition of these plant mixtures. Because of its stability and ease of application, potassium iodide was first used to free the hydrocyanic acid for analysis in this study. The results appeared to be satisfactory for the greater number of plants, but a serious difficulty arose with fresh sorghums. With these samples a cloudy precipitate formed in the second and later fractions of the distillate, making titration impossible, and solid matter of a greenish-black color accumulated in the coils of the Friedrichs condensers used. It was impracticable to filter the precipitate from the distillates so that, although the greater portion of the hydrocyanic acid might be distilled into the first distillate and be determined without difficulty, it was not possible to estimate accurately the small fraction that distilled later.

Study of the interfering substances showed that the precipitate in the distillate consisted of mercuric iodide, and the deposit in the condensers proved to be a mixture of mercurous iodide and metallic mercury. The authors were unable to find a recorded observation of the volatility of mercurous iodide with steam. Distillation of a mixture of potassium iodide and calomel in water led to the formation of a similar deposit in the condenser and of mercuric iodide in the distillate. It was concluded that mercurous iodide, distilling into the condenser, reacts with water to form mercury and mercuric iodide. The latter is washed into the receiver; the former remains with the excess of mercurous compound and may be seen with the aid of a lens in characteristic globules. The mercurous iodide may be dissolved out with potassium iodide solution.

Stannous chloride did not possess this disadvantage, although a minute quantity of mercury compound, presumably calomel, is carried over with the vapors and collects in the condenser as a black film visible after several distillations but in so small a quantity that it does not interfere with the accuracy of the determination or affect the titration in any manner. A series of parallel determinations, potassium iodide being used in one set and stannous chloride in the other, revealed the superiority of the latter as a reagent for the decomposition of mercuric cyanide. The data are reported in table 21, which shows the greater efficiency of stannous chloride except in the case of dried Johnson grass, in which that reagent was distinctly less efficient than potassium iodide. The reason for this discrepancy is not clear.

TABLE 21.—*Recovery of hydrocyanic acid from mercuric cyanide using potassium iodide and stannous chloride*

Plant	Quantity of hydrocyanic acid per 100 g of plant liberated by the use of—		Relative efficiency of stannous chloride to potassium iodide	Plant	Quantity of hydrocyanic acid per 100 g of plant liberated by the use of—		Relative efficiency of stannous chloride to potassium iodide
	Potassium iodide [1]	Stannous chloride [2]			Potassium iodide [1]	Stannous chloride [2]	
	Milligrams	*Milligrams*			*Milligrams*	*Milligrams*	
Hegari	[3] 15	[3] 22	1.47	Feterita	23.8	29.2	1.23
Do	13.6	20.3	1.49	*Prunus serotina*	132.1	133.9	1.01
Johnson grass	100.6	69.3	.69	*Prunus melanocarpa*	186.0	200.7	1.08
Do	[4] 106.7			Arrowgrass	102.8	132.0	1.28
Do	135.5	100	.73				

[1] 4 moles. [2] 2.1 moles. [3] Buffered. [4] 6 moles.

Recovery of hydrocyanic acid from potassium cyanide after treatment of the latter with mercuric chloride was studied with stannous chloride and potassium iodide as reagents. In one case ammonium chloride was added to the stannous chloride in proportion to form the double salt $SnCl_2 \cdot 2\ NH_4Cl$, in an effort to stabilize the tin compound. However, it was found that no advantage in stability was gained, although there was a slight advantage in recovery of hydrocyanic acid. The results are reported in table 22. With potassium iodide the efficiency of recovery was lower than with stannous chloride, and here again mercurous iodide distilled into the condenser and rendered the distillate turbid.

The loss of hydrocyanic acid during these operations suggested that the substance might be decomposed in the distilling flask by hydrochloric acid. A series of experiments was conducted to determine the effect of small quantities of vraious acids on known quantities of hydrocyanic acid under conditions similar to those obtaining in the analytical procedure. The acids, in solution, were added to cold solutions of potassium cyanide. In some cases the mixtures were immediately distilled. In others they were refluxed for from 1 to 2 hours in closed systems, and then the hydrocyanic acid formed was distilled into an alkaline solution and titrated. The results are reported in table 23.

These results indicate that in the ordinary course of analysis some of the hydrocyanic acid is decomposed during the process of distillation and that the value obtained will be low. Bishop (*6*) reports losses ranging from 3 to 5 percent on distilling mixtures of potassium cyanide and dilute acids. Apparently the presence of mercuric chloride or its reaction products and of stannous chloride in the system does not seriously increase the loss from this source.

TABLE 22.—*Recovery of hydrocyanic acid from mercuric cyanide by the use of various reagents*

Reagent	Hydrocyanic acid			Reagent	Hydrocyanic acid		
	Present	Recovered			Present	Recovered	
	Milligrams	*Milligrams*	*Percent*		*Milligrams*	*Milligrams*	*Percent*
Stannous ammonium chloride ($SnCl_2.2NH_4Cl$)	232.1	229.45	98.9	Stannous chloride (excess)	240.5	235.2	97.8
Stannous chloride (1 mole)	239.1	233.6	97.7	Potassium iodide (excess) [1]	240.5	228.1	94.8
Stannous chloride (2 moles)	239.1	234.7	98.2				

[1] Distillate turbid.

TABLE 23.—*Recovery of hydrocyanic acid after heating with 0.5 percent of different acids*

Acid	Time of distillation	Hydrocyanic acid		
		Present	Recovered	Lost
	Minutes	*Milligrams*	*Milligrams*	*Percent*
Oxalic	45	59.24	57.84	2.37
Oxalic (refluxed 1 hour)	45	59.35	40.88	31.13
Phosphoric	45	59.24	55.55	6.23
Sulphuric	40	59.30	55.65	6.15
Sulphuric (refluxed 1.5 hours)	45	59.19	41.56	29.76
Tartaric (refluxed 2 hours)	45	59.33	40.88	31.10
Do	40	59.31	55.44	6.52

SUMMARY AND CONCLUSIONS

Fresh cyanogenetic plants stored at ordinary temperatures without preservatives lost 13 to 83 percent of their hydrocyanic acid in 1 to 6 days.

When stored at refrigerator temperatures for 1 to 5 days, fresh spur feterita yielded as much hydrocyanic acid as before storage, but from one-third to one-half less than was obtained by 24-hour maceration of nonrefrigerated plants. Maceration of refrigerated plants leads to great loss of hydrocyanic acid.

When fresh plants were stored in water with the addition of chloroform, with or without added alcoholic potassium hydroxide or water, losses up to 67 percent occurred in all but two samples. With hegari chloroform was effective as a preservative for 4 days in one sample.

Tests with acid solutions usually resulted in rapid loss of hydrocyanic acid. Salicylic acid preserved most effectively of the acids tested.

Tests with alkaline solutions resulted in losses of from 32 to 96 percent. Organic bases, such as aniline, *p*-toluidine, and pyridine, were more effective. The losses noted in alkaline solut ons appear to be due to destruction of hydrocyanic acid rather than to inhibition of cyanogenesis.

Alcohol in concentrations of 10, 15, 20, and 25 percent preserved for 3 to 7 days with losses up to 8 percent. After a week the losses were larger until, after 7 to 8 weeks, 20 percent of the hydrocyanic acid had been lost. Alcoholic preservation cannot be relied on to

develop the maximum yield of hydrocyanic acid potential in the plant. Alcohol in concentrations of 50 and 95 percent inhibited cyanogenesis.

Mercuric chloride in water solution proved to be an excellent preservative when used in the proportion of 1 percent by weight for fresh plant. Specimens so preserved and stored for 6 months have shown no loss of hydrocyanic acid. Lower concentrations of mercuric chloride either did not preserve or led to variable results. A concentration of 2-percent mercuric chloride preserved dried plants reasonably well for 3 months. With dried sorghums and for longer periods, a higher, undetermined concentration would be required.

Plants preserved with mercuric chloride generally yielded more hydrocyanic acid than when not so preserved except in certain cases in which the experiments may have been terminated before the maximum yield of hydrocyanic acid was obtained.

In the concentrations used mercuric chloride retarded but did not stop enzyme action in cyanogenetic plants. Buffering did not counteract this effect and the addition of chloroform did not accelerate enzymolysis. The addition of enzyme greatly accelerated the rate of hydrocyanic acid formation in the presence of mercuric chloride.

The rate of formation of hydrocyanic acid in cyanogenetic plants is not uniform. A drop in hydrocyanic acid yield begins about 8 to 10 hours and continues for several hours before the yield again reaches the level observed after the first 8 hours. The period that elapses between the beginning and recovery lasts for 10 to 12 hours. This phenomenon is very characteristic and has been noted in all the plants studied.

The figure for the hydrocyanic acid content of plants determined after maceration with water or diluted alcohol is the resultant of two processes—cyanogenesis and conversion of freed hydrocyanic acid into other compounds, often referred to as destruction of hydrocyanic acid. The value obtained may be considerably below the true figure.

Large quantities of hydrocyanic acid are converted into other compounds when hydrocyanic acid is heated with very dilute acids. This results in an unavoidable error in the determination of hydrocyanic acid in plants.

The optimum temperature for storage of samples preserved with mercuric chloride solution, as between a refrigerator at 9° C., the laboratory at 25°, and an incubator at 37°, was found to be 25°.

An analytical technique for recovering hydrocyanic acid from samples preserved with mercuric chloride was developed. To liberate hydrocyanic acid from mercuric cyanide, stannuous chloride was found to be preferable to potassium iodide except in one instance.

Mercurous iodide appears to be volatile with steam and deposits in condensers where it reacts with water to form mercuric iodide and metallic mercury. The mercuric iodide may render distillates turbid and interfere with the end point in titration.

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RELATION OF ATMOSPHERIC CONDITIONS TO ENLARGEMENT RATE AND PERIODICITY OF WINESAP APPLES [1]

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INTRODUCTION

Since the early demonstrations of periodicity in plant growth by Sachs (*20*),[2] in 1874, many investigators have studied this phenomenon in various species of plants but comparatively few have directed their attention to tree fruits and vegetables. It was observed by MacMillan (*18*) that the enlargement of the potato tuber over a 24-hour period was not continuous but rhythmic and that the growth-rate maxima were followed by periods of slower growth or by complete cessation of enlargement. MacMillan believed that this periodic growth of the tuber was influenced by the periodic growth of the aerial portions of the plant. D. F. Fisher,[3] in 1920, obtained continuous measurements of the diameter increase of apples over 24-hour periods, and from his studies concluded that during the summer the enlargement of apples takes place mainly at night.

In recent years the rate of fruit enlargement has been used as an index of tree response to various factors affecting tree growth and behavior. Furr and Magness (*12*), working with apples, Aldrich and Work (*1*) with pears, Hendrickson and Veihmeyer (*16*) and Cullinan and Weinberger (*9*) with peaches, and many others have studied response to soil-moisture variations by measuring the circumference of the fruits at frequent intervals during the growing season. Aldrich and Work (*1*) also included in their study the influence of evaporating power of the air on growth of pears by noting the difference in circumference growth of fruit during periods of high and low evaporating power.

The value of such fruit measurements as an aid in interpreting environmental influences in the field is quite evident, and they probably will be employed to an even greater extent in the future than they have been in the past. In these researches some consideration has been given to the periodicity of fruit enlargement by taking fruit measurements at a specified time of day. Any error resulting from periodic fluctuations in rate of growth would probably be small if the intervals between measurements were 2 days or more. However, it is quite desirable in some studies to take daily measurements, in which case periodicity might become quite important.

The present study was undertaken to determine the daily enlargement rate and periodicity in growth of Winesap apples in the Wenatchee Valley, Wash., and the effect, if any, of atmospheric influences. One objective of the experiment has been the possible application of these findings to other fruit-growth studies now contemplated.

[1] Received for publication October 1, 1937; issued August 1938.
[2] Italic numbers in parentheses refer to Literature Cited, p. 123.
[3] Unpublished data in possession of writers, 1920.

METHODS AND MATERIALS

To estimate the rate of fruit growth during relatively short intervals, an apparatus was desired which would give a continuous record and which would be sufficiently sensitive to measure small changes in enlargement. An instrument was finally devised which fulfilled both of these requirements. Two of these instruments were used in the orchard to record fruit growth during the last 3 months of the 1936 growing season at Wenatchee, Wash. The apparatus was set up by encircling an apple at its greatest circumference with a strip of brass tape 0.051 mm thick, 5 mm wide, and 30 cm long. One end of this tape was widened and slotted to permit the other end to pass through. The narrow end was held in a fixed position and the broad slotted end attached to a modified hygrograph assembly. A standard hygrothermograph clock with weekly charts was used to register the movement of the pen arm. Expansion of the apple caused the tape to pull against the magnification levers of the pen arm, and the enlargement was thus recorded. A piece of 1¼-inch angle iron 20 inches long, bolted to the bottom of the case, afforded a base for clamps and also served to increase the rigidity of the assembly. The apple under observation was held by a large light-spring clamp, the jaws of which were cushioned with soft rubber tubing. It was found necessary to hold the apple rather firmly but without restricting growth.

The instruments were placed under the tree on a table that was set securely in place and staked to the ground. The main branch bearing the apples was solidly braced, and the smaller limbs were carefully held in position with clamps padded with cotton. It was essential, of course, to minimize movements of the branches by wind or other agencies in order to prevent transmission of such disturbances to the recording pen. No difficulty was experienced from this source in the present study. The apparatus as it appeared in the orchard is shown in figure 1.

Certain inherent errors, obviously present in studies of this character where physical methods are employed, may in some cases seriously limit the field of adaptability. In the present study, for example, it seemed necessary to determine and take into consideration the variations that might arise from the effect of temperature changes on the recording mechanism. The thin metal tape encircling the apple was found to have a coefficient of thermal expansion of 20×10^{-6}. The instruments were standardized both in the laboratory and in the field by substituting a porcelain sphere for the apple and noting corrections necessary for temperature fluctuations. This sphere had a coefficient of expansion so low that it was considered negligible. Within the range of temperatures prevailing in this experiment, corrections for thermal expansion did not materially change the values on the original chart.

A limitation of a more serious nature became evident as the study progressed. The pressure of the tape on the apple, exerted by the small springs in the connected lever mechanism, although very light, appeared to retard fruit enlargement slightly. To correct for this apparent error, each time the chart was changed the calibration of the instrument was checked by measuring the apple with a hand tape. This tape was graduated to millimeters but with care could

be read to one-half millimeter. To further check the relative growth rate, 25 apples on the same tree, selected for uniformity of size, were measured daily with the tape. It was found that the average daily enlargement of these apples followed closely that of the apple in the instrument. The apparatus proved quite sensitive to changes

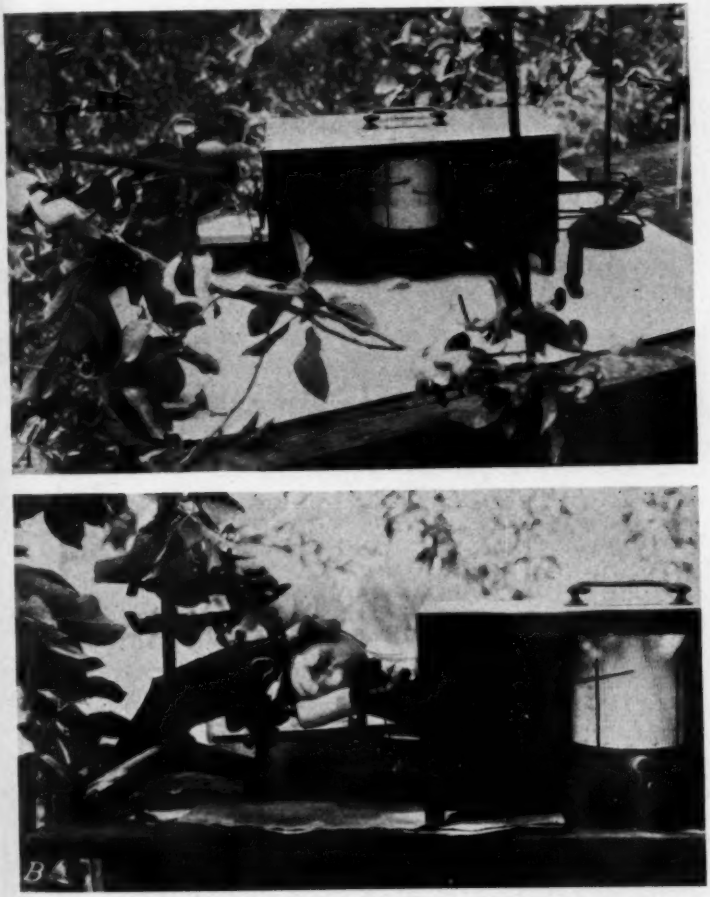

FIGURE 1.—Apparatus used for obtaining a continuous record of growth of apple fruit on the tree: *A*, Clamps holding branches; *B*, apple in position, encircled by brass tape attached to recording mechanism.

in fruit enlargement. A space equivalent to 1° on the 1072–D thermograph chart used represented an average volume increase in the apple of from 0.05 to 0.10 cc, depending on the calibrations of the instrument. In the conversion of circumference measurements to volume, the apple was assumed to be a sphere.

The experiment was carried on from July 20, after extension growth had ceased, until harvest on October 11. With the aid of the instruments a continuous record of fruit growth was obtained during this period. Atmospheric temperatures were recorded simultaneously on the same chart. The relative evaporating power of the air was estimated by the use of standardized white atmometers. One atmometer was read at 4-hour intervals beginning at 8 a. m. and ending at 8 p. m.; the other was read daily at 4 p. m. Air movement was measured by means of a small fan-type anemometer, mounted with a V-type vane and balanced on an enclosed ball bearing. Its response to slight changes in wind direction was rapid and positive. Anemometer and atmometer readings were taken at the same time.

The trees on which the experiment was performed were large, vigorous Winesaps about 30 years of age, carrying a heavy load of fruit. They were growing in a deep, moderately light clay-loam soil with excellent drainage. The soil was irrigated frequently to insure a high moisture supply at all times. The moisture percentages at three soil horizons, with their respective wilting percentages and dates of irrigation, are given in table 1.

TABLE 1.—*Soil-moisture determinations on experimental plot for study of relation of growth of Winesap apples to evaporating power of the air, air movement, and atmospheric temperature, 1936* [1]

Sampling date	Moisture in soil horizon [2]			Sampling date	Moisture in soil horizon [2]		
	1 and 2 feet	3 and 4 feet	5 and 6 feet		1 and 2 feet	3 and 4 feet	5 and 6 feet
	Percent	*Percent*	*Percent*		*Percent*	*Percent*	*Percent*
July 28	19.2	12.5	9.5	Aug. 29	19.3	13.5	12.2
Aug. 1	13.8	11.0	13.2	Sept. 2	.....	12.2	10.5
4	16.4	9.6	12.8	7	17.0	11.7	9.5
8	18.5	12.9	14.8	11	14.9	12.7	14.4
12	19.8	11.6	11.7	18	17.7	12.9	11.8
17	16.0	12.2	10.3	23	13.9	10.2	9.5
21	15.2	12.1	11.1	28	16.8	12.5	11.0
25	14.1	11.5	10.4	Oct. 5	13.9	10.2	11.0

[1] Irrigations: July 24–27; Aug. 5–7, 26–28; and Sept. 15–17.
[2] Wilting points, from laboratory determinations with sunflower plants: 1 and 2 feet, 7.8 percent; 3 and 4 feet, 6.1 percent; 5 and 6 feet, 5.5 percent.

EXPERIMENTAL RESULTS

Examination of the chart records gave early indications of a definite daily periodicity in the enlargement of apples. The rhythm of growth from day to day, however, was not always uniform, nor did it remain the same throughout the season. During the first 4-week period, July 20 to August 16, on clear, bright days, enlargement of the fruit took place almost entirely between 7 p. m. and 10 a. m., the maximum rate occurring between midnight and 2 a. m. This is illustrated by figure 2, which shows that on August 4, 5, and 6 the apples made no perceptible growth between 10 a. m. and 7 p. m.; in fact, the August 6 record indicates very slight shrinkage of the fruit between 3 and 7 p. m. This shrinkage usually was found to occur on days when the evaporating power of the air was relatively high. The loss of water from white atmometers on August 4, 5, and 6 was 26.3, 36.9, and 63.8 g, respectively. Corresponding volume increases of the fruit were

2.03, 1.68, and 1.52 cc. Although the maximum temperatures for August 4 and 5 were considerably higher than that of August 6, the evaporating power of the air was lower, indicating that high evaporating power of the air exerts a greater retarding influence on fruit growth than high temperatures alone, although the two are often closely associated. Reference is also made to table 1, indicating that the soil moisture was not limiting at this time. Water was applied to the soil from August 5 to 7. The fact that the enlargement rate of the fruit was greater before than during irrigation suggests that in this experiment reduction in growth was caused by factors other than lack of available soil moisture.

The August 2 growth curve, which is characteristic of fruit enlargement on days of extremely low evaporating power, is shown in figure 3.

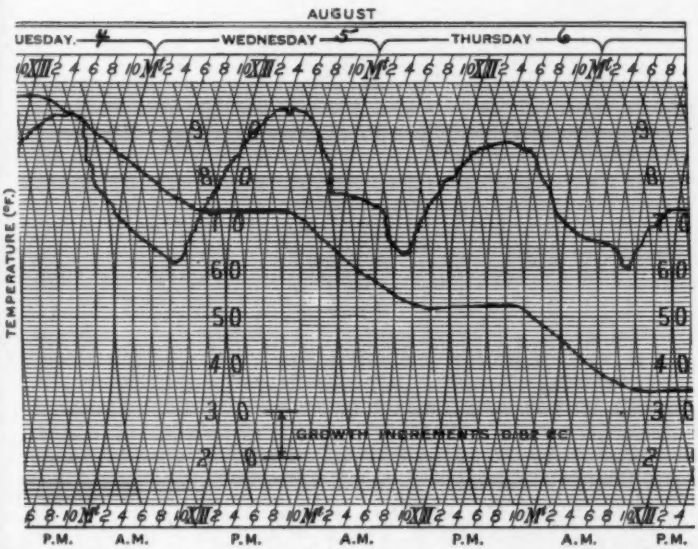

FIGURE 2.—Fruit-growth chart showing periodicity in time and rate of growth of an apple on August 4, 5, and 6. The growth curve is the light line taking a course toward the bottom of the chart. Temperatures are shown by the heavy line. For hours of growth, read bottom; for hours of temperature, read top, deducting 2 hours.

There was little perceptible air movement throughout the day, and the 24-hour water loss from white atmometers was only 18.9 g; the volume increase of the fruit was 2.22 cc. As shown on the chart, fruit enlargement was continuous throughout the 24 hours under these conditions, although the rate was somewhat less during the day than it was at night.

As the season progressed it became quite evident that a change was taking place in the diurnal periodic cycle of growth. In the second 4 weeks of the experiment, August 17 to September 13, the average growth rate and the total amount of growth per day were less than in the previous 4 weeks, and the apples tended to make a slightly greater percentage of their growth during the daylight hours. A

typical curve from the instrument chart illustrating growth during this period is given for August 20 and 21 in figure 4.

Figure 5 shows the growth curves for September 23 and 24, which characterize in general the daily periodicity for the third 4-week period, September 14 to October 11. It is readily observed that the total growth on these days is considerably less than is shown in figures 2, 3, and 4, and also that a different periodicity is exhibited. The period of most rapid enlargement occurred during the morning daylight hours, reaching a maximum between 10 a. m. and 12 noon. In general, during the entire 4 weeks there was a tendency to show some enlargement throughout the 24 hours; however, a fairly well-established minimum prevailed from 4 p. m. to 12 midnight. The average total growth is considerably less than that of the two previous periods.

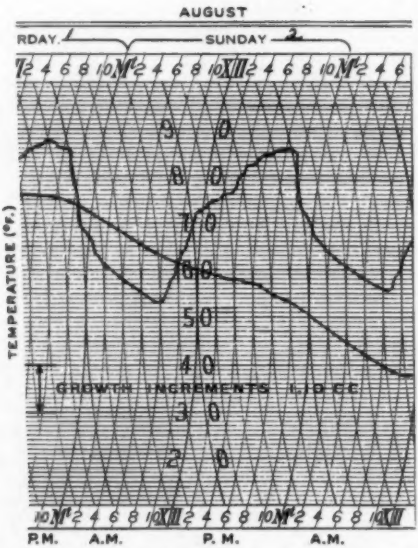

FIGURE 3.—Fruit-growth chart showing periodicity in time and rate of growth of an apple on August 1 and 2. The growth curve is the light line, and temperatures are shown by the heavy line. For hours of growth, read bottom; for hours of temperature, read top, deducting 2 hours.

Variations in average diurnal rate of enlargement of apples in each of the three seasonal divisions are diagrammatically summarized in figure 6. These divisions or periods were arbitrarily selected to demonstrate best the periodic drift over the 3 months' duration of growth. Rate of growth is given in terms of volume increase in cubic centimeters, at 2-hour intervals. The average enlargements per day for the three periods are as follows: July 20 to August 16, 1.89 cc; August 17 to September 13, 1.66 cc; September 14 to October 11, 1.16 cc.

Perhaps the most striking feature of these curves is the shift in time and magnitude of the growth climacterics as the fruit advances toward maturity. The greatest contrasts are found between the first

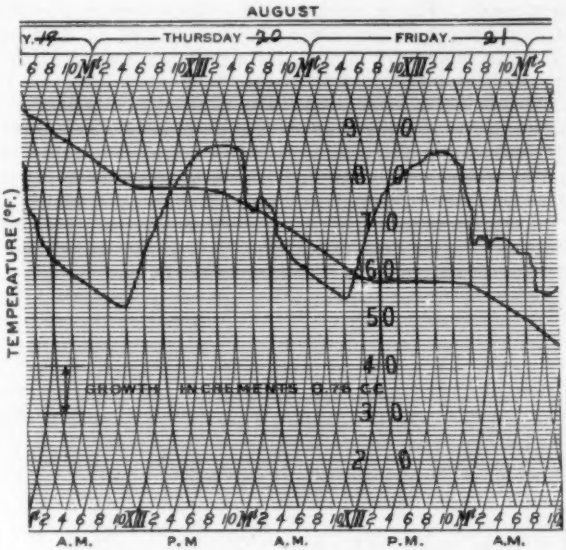

FIGURE 4.—Fruit-growth chart showing periodicity in time and rate of growth of an apple on August 19, 20, and 21. The growth curve is the light line, and temperatures are shown by the heavy line. For hours of growth, read bottom; for hours of temperature, read top, deducting 2 hours.

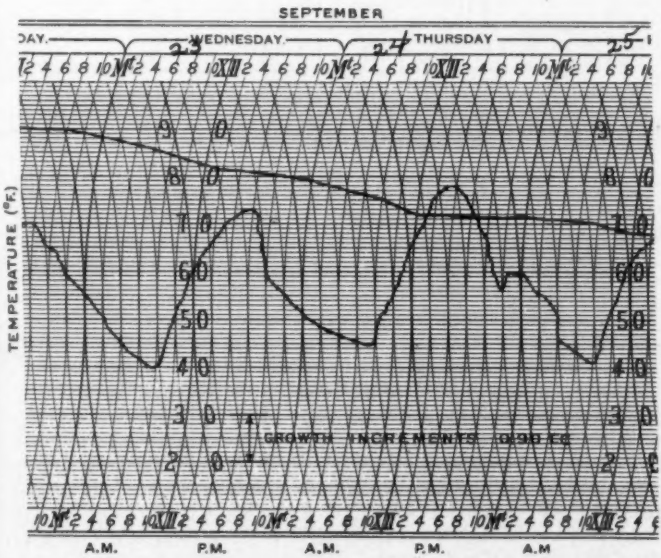

FIGURE 5.—Fruit-growth chart showing periodicity in time and rate of growth of an apple on September 23 and 24. The growth curve is the light line, and temperatures are shown by the heavy line. For hours of growth, read bottom; for hours of temperature, read top, deducting 2 hours.

and third periods. For example, rapid enlargement in the first period takes place within the same hours as does minimum enlargement in the third. Likewise, the hours of maximum growth in the third period occur within the time range of minimum growth in the first.

A relationship perhaps worthy of special emphasis is indicated on this chart (fig. 6). Throughout the 24 hours only two points on each of the three curves coincide in showing both a uniform and a minimum growth. These are found at 4 and 6 p. m. and represent the average lowest rates of enlargement over the three periods. This time period may therefore be considered as having a rather well-established minimum growth rate for this portion of the growing season and might be designated as the "mean diurnal minimum."

The average seasonal enlargement of the 25 hand-measured apples used as a check on the apple in the instrument is shown in figure 7.

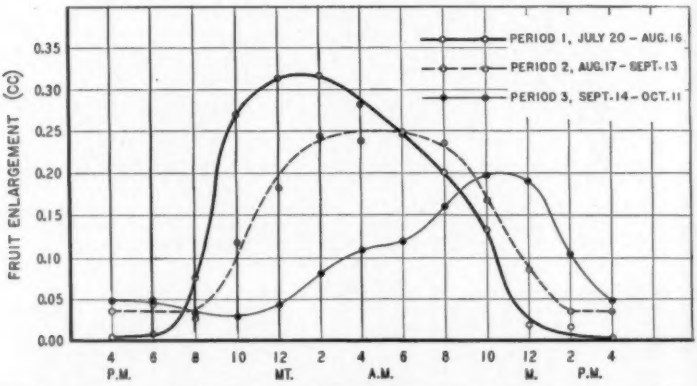

FIGURE 6.—Average diurnal enlargement rate of Winesap apples during the 1936 season, showing change in daily periodicity for three consecutive periods.

This curve shows a departure from straight-line growth early in September, when a decline of approximately 22 percent took place. This deviation from a uniform enlargement may be characteristic of late varieties such as Winesap. Oldenburg (*12*), an early-maturing variety, did not show this change in growth up to the time of harvest.

Attention is again directed to the effect of evaporating power of the air on the daily growth rate and periodicity of fruit enlargement, as mentioned above and as illustrated in figures 2 and 3. In table 2 the average daily enlargement of fruit, mean temperature, and air movement, occurring with different rates of evaporating power of the air, are given for the three 4-week periods in 1936. In preparing this table the days of each period were arbitrarily divided into four groups according to the evaporating power of the air, and each day's growth, mean temperature, and air movement were placed in their respective evaporation groups and averaged. A very definite negative correlation can be seen between average daily fruit growth and evaporating power of the air. Of the factors studied, including evaporating power, air movement is apparently an especially important one and follows closely the same relationship to growth in the last two periods but not in the first. The deduction, therefore, is that in this experiment air

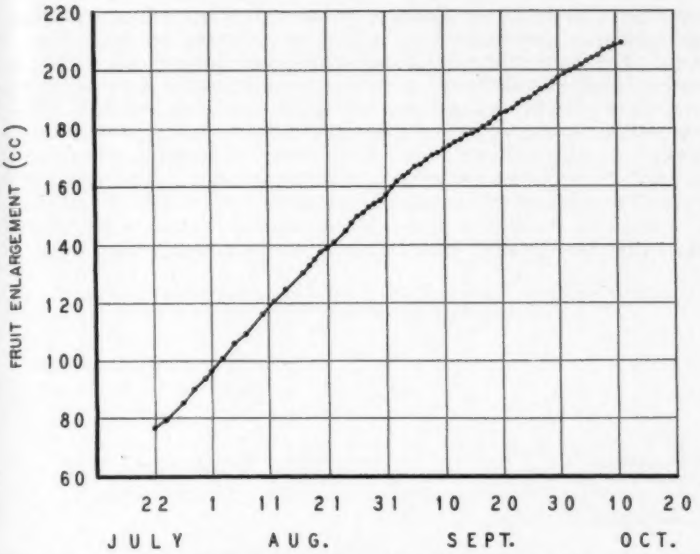

FIGURE 7.—Average seasonal enlargement of 25 Winesap apples obtained from hand-tape measurements.

TABLE 2.—*Relation of average daily enlargement of Winesap apples, air movement, and mean temperature, to four levels of evaporating power of the air, 1936*

Evaporating power of air (grams H_2O per day)	First period (July 20 to Aug. 16)			Second period (Aug. 17 to Sept. 13)			Third period (Sept. 14 to Oct. 11)		
	Enlargement of fruit per day	Linear air movement per day	Mean temperature	Enlargement of fruit per day	Linear air movement per day	Mean temperature	Enlargement of fruit perday	Linear air movement per day	Mean temperature
	Cc	*Feet* [1]	° *F.*	*Cc*	*Feet* [1]	° *F.*	*Cc*	*Feet* [1]	° *F.*
40 and above	1.79	207	75.4	1.42	212	66.1			
30 to 40	1.99	104	72.6	1.49	148	63.8	0.83	165	61.7
20 to 30	2.15	39	74.7	1.77	41	65.3	1.17	114	59.0
Less than 20				1.93	50	61.3	1.23	43	54.1

[1] In thousands.

movement was probably the dominating factor of those affecting the daily enlargement rate of apples. The correlation coefficients for fruit enlargement, evaporating power of the air, air movement, and temperature are shown in table 3.

The possible association of atmospheric influences with daily periodicity of fruit enlargement is indicated in table 4. Here the 24-hour day is divided into four intervals, and corresponding average hourly growth rates, mean temperatures, evaporating power of the air, and air movements are given for comparison. Examination of these data reveals that, with the exception of the 4 p. m. to 8 a. m. interval in the third period, the hours of relatively high growth rate are invariably associated with either low evaporating power of the air or low air

movement, or both; conversely, hours of low growth are associated with either high evaporating power or increased air movement, or both. This indicates a relationship between growth rate and evaporating power for divisions of the day similar to that between average total daily growth and evaporating power as shown in tables 2 and 3. It is rather significant that the lowest fruit-growth rates occur between 12 noon and 8 p. m., and that the highest evaporating powers of the air and air movements also occur during this time. The fact that the greatest movement of air takes place in the latter half of this period, between 4 p. m. and 8 p. m., is noteworthy, for it is within these hours that the mean diurnal minimum evaporation is found.

TABLE 3.—*Correlation coefficients for fruit enlargement, evaporating power of air, air movement, and temperature (from data in table 2)*

Item	Evaporating power of air		Air movement		Temperature	
	r	*P*[1]	*r*	*P*[1]	*r*	*P*[1]
Fruit enlargement	−0.95	<0.01	−0.91	<0.01	−0.76	0.01
Evaporating power of the air			+.97	<.01	+.65	.03

[1] Fisher's "probability" (*z* test used) (*11*).

TABLE 4.—*Relation of average hourly enlargement of Winesap apples, evaporating power of the air, air movement, and mean temperature, to four time periods of 24-hour day, 1936*

Time intervals	First period (July 20 to Aug. 16)				Second period (Aug. 17 to Sept. 13)				Third period (Sept. 14 to Oct. 11)			
	Enlargement of fruit per hour	Evaporating power of air (H_2O per hour)	Linear air movement per hour	Mean temperature	Enlargement of fruit per hour	Evaporating power of air (H_2O per hour)	Linear air movement per hour	Mean temperature	Enlargement of fruit per hour	Evaporating power of air (H_2O per hour)	Linear air movement per hour	Mean temperature
	Cc	*Grams*	*Feet*	° *F.*	*Cc*	*Grams*	*Feet*	° *F.*	*Cc*	*Grams*	*Feet*	° *F.*
8 a. m. to 12 m.	0.038	2.48	6,500	81.0	0.063	1.82	5,300	71.0	0.097	1.00	2,700	65.3
12 m. to 4 p. m.	.005	3.08	5,900	86.4	.018	2.50	7,000	76.9	.038	2.20	5,300	70.1
4 to 8 p. m.	.021	2.32	7,300	81.1	.018	1.75	7,500	67.4	.021	1.20	6,400	58.9
8 p. m. to 8 a. m.	.136	.82	4,200	65.8	.105	.43	2,700	55.5	.045	.13	1,100	47.9

Average temperatures throughout the 24-hour day for each of the three periods of the experiment are shown in figure 8. Approximate hours and temperatures of maximum and minimum rate of fruit enlargement are also indicated. That temperature may be a factor in the changes in seasonal periodicity is evidenced by this chart. It appears significant that the hours of maximum fruit growth in all periods occur within the 50° to 75° F. range regardless of the hour of day or night when this high growth rate occurs. The possible relation of temperature to hours of minimum growth is not so apparent, however. On the curve for July 20 to August 16, minimum enlargement rate is associated with temperatures well above 80°. From August 17 to September 13, or during the second period, the minimum rate broke into temperatures occurring at the time of maximum growth in the first period; while in the final period, September 14 to October

11, both maximum- and minimum-growth hours fell largely within the same temperature limits. This does not necessarily show that low temperatures may not retard fruit enlargement, for in figure 6 the curve representing the period from September 14 to October 11 clearly indicates a retardation in rate of growth during the night and early morning hours when temperature is comparatively low, especially between 4 and 6 a. m. Data from this experiment, however, give no evidence that temperature alone has any pronounced effect on the mean diurnal minimum.

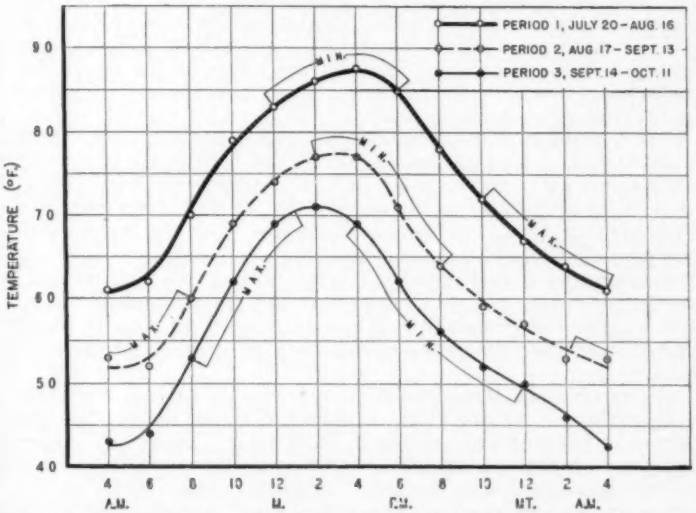

FIGURE 8.—Average atmospheric temperatures through 24-hour day during 1936 season, and approximate hours of maximum and minimum fruit-enlargement rate for three consecutive periods.

Temperature relationships, in contrast with evaporating power and air movement, seem again to be uncertain, and it appears that within the temperature limits of this experiment high temperature exerts an indirect effect on growth of fruit through increasing the evaporating power of the air. That low temperatures may directly limit growth is evidenced in the third period from 8 p. m. to 8 a. m. (table 4). This was the only time during the experimental season that maximum growth was not associated with minimum evaporating power and air movement. It was also the only time that the average temperature fell below the optimum growth range of 50° to 75° F. (fig. 8). Apparently, then, temperature below a certain range is a limiting factor in fruit enlargement, and this may account for the shift in the hours of maximum enlargement in the third period as compared with the first and second (fig. 6).

DISCUSSION

The results reported in this paper are too limited to enable one to generalize with much confidence on the many processes involved in plant periodicity, but they do point to a few environmental factors that seem to be of importance in regulating the periodic enlargement of apples. In this investigation, evidence presented indicates that time and relative rate of fruit enlargement are closely associated with atmospheric conditions. Aldrich and Work (*1*) found that pears showed a reduced growth rate during days of high transpiration, when soil moisture was highly available. Data from the present experiment (figs. 2 and 3; table 2) substantiate these findings and in addition to this reduction of total daily growth by high evaporation show a definite association between the hours of the day when the evaporating power of the air was highest and the rate of growth of the fruit was lowest (table 4).

If one considers that enlargement of apples depends on the transport of water and elaborated substances to the fruit from other portions of the tree, any factor or group of factors interfering with this movement would probably retard enlargement. A high evaporating power of the air would tend to increase water loss from the leaves, and it is thus possible that, during periods of high transpiration accompanied by a saturation deficit in the leaf, transport of materials to the fruit may be retarded and water may actually be withdrawn from the fruit. Upon the restoration of leaf turgor, which usually occurs at night during the summer, a resumption of fruit enlargement takes place.

It is not within the scope of this report to discuss the effect on transpiration or translocation of each individual factor that goes to make up the evaporating power of the air. However, under the conditions of this investigation there is rather conclusive evidence that air movement was the most important single factor studied in increasing the evaporating power of the air, as measured by water loss from white atmometers. If an attempt is made to account for increased transpiration in terms of air movement the writers are not wholly in agreement with the findings of many investigators; however, the experimental evidence in regard to the influence of wind on transpiration and plant response is itself somewhat confusing. Briggs and Shantz (*4*, *5*) found that, of the physical environmental factors studied, wind velocity showed the lowest correlation with transpiration. They considered that only 2 to 6 percent of the transpirational loss could be attributed to the action of wind.

The apparently conflicting evidence regarding factors that affect transpiration in the field may in some cases be traced to climatic differences in the location of the experiment. It is possible that under certain atmospheric conditions air movement, as compared with other influences, may have but a slight effect in increasing transpiration. Studies in the field, at best, are complicated by many opposing and interacting factors and the interpretations frequently reflect only the forces most active at a particular time.

The terrain adjacent to the orchards of the Wenatchee Valley is arid during the summer months. In the morning and early afternoon the surrounding desert land and rocky hillsides absorb considerable heat from the sun's rays. Subsequent air movement in

the afternoon tends to carry into the cooler orchards the resultant hot, dry atmosphere from these outside areas. Under these conditions air movement, in addition to exerting a possible direct influence in increasing water loss from the leaves, may also produce the same result by bringing about a combination of forces, such as lowered humidity and increased atmospheric and leaf temperatures.

Knight (*17*) found that a wind velocity of 0.26 mile per hour caused a 50-percent increase in transpiration rate over that in still air. Copeland (*8*) in the Philippine Islands found that transpiration of the coconut palm was four times as great in a wind with a velocity of 5 miles per hour as in a calm. Others (*6, 19, 21*), however, have concluded that transpiration was increased up to a limited air velocity, above which there was a pronounced depressing effect. Martin and Clements (*19*), using motor-driven fans on sunflowers, found a striking decrease in leaf and stem development, dry weight, and total transpiration with increasing wind velocities. These findings suggest that air movement may bring about physiological changes, correlated with increased transpiration, which may in themselves exert a retarding influence on fruit enlargement.

Recently Audus (*2*), and also Godwin (*13*), studying the effect of various treatments on the respiration rate of leaves, reported a twofold to threefold increase in respiration resulting merely from bending or rubbing the leaves lightly past one another. After the treatment, respiration gradually decreased, but did not reach normal until after about 60 hours. From the results obtained by these investigators it appears that respiration of apple leaves may be increased by the motion or rubbing action caused by wind to the extent of retarding fruit enlargement. Heinicke and Childers (*14*) offer further evidence in this direction by showing that during the daytime a high rate of respiration in apple leaves greatly reduced the rate of photosynthesis.

These possibilities, either singly or collectively, point to a partial explanation of the effects of air movement on fruit growth. They also suggest that a reduction in diurnal fruit enlargement may not be due entirely to a temporary delay in translocation, but that some of the elaborated materials may be permanently lost to the fruit. It has been observed that apple trees in localities characterized by constant winds are usually stunted in growth and seldom highly productive. Blanchard (*3*) found a 5- to 7-percent increase in yield of lemons and a 3-percent increase in size of tree when the grove was protected from wind.

If the enlargement rate of apples through the season is adversely affected by high evaporating power of the air, there would probably be evidence of the same influence on daily periodic growth. Data supporting this assumption are shown in table 4. An important factor to be considered in this connection is the time of day, throughout the season, when fruit-enlargement rate is likely to exhibit the least variability. In the present experiment such a time was found between 4 and 6 p. m., when the average minimum growth rate of apples for the experimental season occurred (fig. 6). Daily fruit measurements taken at this time, therefore, will probably be influenced least by periodic growth variation. In this work this period has been designated as the "mean diurnal minimum." It must be recognized, however, that this may hold true only under conditions in the Pacific Northwest or perhaps within even narrower geographic limits.

To account fully for the change in diurnal periodicity from one seasonal period to the next is rather difficult with the data at hand. Shifting of the daily maxima of fruit enlargement both in time and magnitude as the fruit approaches maturity is undoubtedly brought about by fundamental changes in metabolism, some of which, as have been shown, are probably influenced by environmental conditions. Other changes might be the result of hereditary characteristics peculiar to the species or variety. That pronounced periodicity can be induced in plants by variation in the amount of daily illumination is well recognized, and this may also play an important part in the seasonal periodicity of fruit enlargement.

During the course of this investigation, for example, daily illumination as measured from sunrise to sunset decreased from 15½ to 11 hours. According to Heinicke and Hoffman (*15*), such a reduction in length of day would decrease photosynthetic activity of the leaf. This, accompanied by lowering light intensities as the season progresses (*7*), might result in a lower rate of fruit growth.

The growth curve for the third period (fig. 6) is of particular interest in the light of recent researches by Curtis and Herty (*10*) on the effect of relatively low temperatures on translocation. These authors found that chilling the petioles of bean leaves to temperatures between 0.5° and 4.5° C. greatly reduced transport of carbohydrates from the leaves, and that maintaining the petioles at 7° to 11° allowed greater translocation, but less than at temperatures of 17° to 24°. The fruit-enlargement curve for this third period shows a response to temperature variations similar to that described above. The relatively low average night temperatures of this period (6.5° to 10°) fall within one of the temperature ranges used by Curtis and Herty (*10*), and it appears significant that the hours of slow fruit enlargement coincide with these comparatively low temperatures. The rather sharp change in slope of the curve (fig. 6) from 4 to 6 a. m., where the lowest average temperatures were encountered, tends to emphasize this relationship.

SUMMARY AND CONCLUSIONS

By means of continuous-recording instruments, checked by daily hand-tape measurements, a study was made of the relationship between atmospheric conditions and the time and rate of enlargement of Winesap apples.

Reduction in the enlargement rate of fruit was closely associated with comparatively high evaporating power of the air, and under the conditions of this investigation air movement appeared to be the dominant factor in increasing this evaporating power.

Atmospheric temperatures probably have an important influence on the enlargement rate of apples. The effect of high temperatures in retarding growth may be exerted indirectly by increasing the evaporating power of the air. There is some indication that temperatures below 50° F. may directly limit fruit growth.

A pronounced daily periodicity was found in the time and rate of fruit enlargement, and this periodicity seemed to be influenced by the evaporating power of the air. Hours during which average growth was at a minimum were characterized by high evaporating power of the air and increased air movement.

The daily curve of growth did not remain constant throughout the investigation but showed a distinct seasonal shift in maximum and minimum rates. From July 20 to August 16 the highest rate of fruit enlargement occurred between 10 p. m. and 4 a. m.; from August 16 to September 13 the maximum rate was between 2 and 8 a. m.; while in the third period, September 14 to October 11, it was found from 8 a. m. to 1 p. m. The average daily growth for these three periods was 1.89, 1.66, and 1.16 cc, respectively.

The least fluctuation in daily growth rate throughout the experiment was found in the minimum growth range, between 4 and 6 p. m. This period has been termed the "mean diurnal minimum" of fruit enlargement. Daily fruit measurements taken at this time will therefore be less affected by both diurnal and seasonal periodic variations in growth than at any other time of the 24 hours.

Some possible physiological effects of air movement and evaporating power of the air on the retardation of fruit enlargement are discussed.

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THE SOLUBILITY OF CASEIN IN SALTS OF CERTAIN ORGANIC ACIDS, AND ITS FRACTIONATION BY MEANS OF THESE ACIDS[1]

By RALPH H. MCKEE, *professor of chemical engineering, Columbia University,* and STEPHEN P. GOULD, *assistant chemist, Division of Dairy Research Laboratories, Bureau of Dairy Industry, United States Department of Agriculture*[2]

INTRODUCTION

The study of casein, the principal protein in milk, is being given increasing attention in research laboratories, not only because of the promising new ways in which it may be used in industry, but also because it affords a suitable material for the study of fundamental problems in protein chemistry.

Strong acid and alkaline reagents have generally been used as solvents for casein, but they are not entirely satisfactory. Accordingly, the writers undertook experiments to determine the degree of solubility of casein in solutions of certain salts of organic acids. Nearly neutral aqueous solutions having a pH value of 4.6 (the isoelectric point of casein) were used in these experiments, since very little research work had been done with such solutions as compared to the work done with strong acids and bases.

DEFINITION OF TERMS

The term "casein" as used here means "acid casein" as distinct from rennet casein. Acid casein might be further defined as the phosphoprotein, or aggregation of phosphoproteins, precipitated from skim milk by acid at a pH value of 4.1 to 4.6. The phosphorus content, as determined by different investigators, varies from 0.71 to 0.88 percent.

Rennet casein is evidently a calcium caseinate—calcium phosphate complex—and is not nearly so pure as acid-precipitated casein. Rennet casein is relatively unimportant commercially; approximately 97 percent of all the casein produced is acid casein. Acid casein is the product commonly known in the industry and in the scientific literature as "casein."

By "solubility of casein" is meant the solubility at the isoelectric point rather than that resulting from chemical combination, as, for example, in borax solution where sodium caseinate is formed and dissolved.

The term "solution" in this paper refers both to crystalloidal and to colloidal solutions.

[1] Received for publication January 29, 1938; issued August 1938. This paper was presented in part before the meeting of the American Chemical Society, Chapel Hill, N. C., April 1937. It is condensed from the dissertation submitted by Stephen P. Gould in partial fulfillment of the requirements for the degree of doctor of philosophy in the Faculty of Pure Science, Columbia University.

[2] The writers acknowledge the generous cooperation and helpful advice of L. A. Rogers, Chief, and E. O. Whittier, senior chemist, Division of Dairy Research Laboratories, Bureau of Dairy Industry.

SOLUBILITY OF ACID-PRECIPITATED CASEIN

EARLIER INVESTIGATIONS DEALING WITH SOLUBILITY OF CASEIN

Much of the earlier work on casein solvents or plasticizers in the organic field was done in connection with casein plastics. Goldsmith (*12, 13, 14*) [3] in 1907 found that naphthol and other phenolic compounds and urea would "convert" casein to the "thermoplastic" state. This he believed was due to "the solvent action of the converting agent." Phenolsulphonic acid, tartaric acid, aniline, borax, and naphtholsulphonic acid (*28*) are also mentioned in the early patent literature of plastic casein.

Cohn (*7, 8*) reported the solubility of casein in water at its isoelectric point as 0.11 g per liter at 25° C. Euler and Bucht (*9*) determined quantitatively the solubility of casein in tartaric acid, sodium potassium tartrate, and chloracetic acid solutions. They determined the amount of casein in the solution by precipitating it with sodium acetate solution or acetic acid, filtering, and drying in a vacuum desiccator. At the Carlsberg Laboratories in 1925 Kondo (*19*) examined the effect of hydrochloric acid on casein. By formol titration, he showed that there was no hydrolysis at 18° C.

Agthe (*1*) records citric acid, sodium naphthylaminosulphonate, and potassium butyrate as agents for making casein soluble.

In view of the fact that an acid that considerably swells casein will usually dissolve it, the work of Isgarischew and Pomeranzewa (*16*) is of great interest. They tested the swelling action of casein in solutions of 23 organic acids. The greatest swelling action was shown by chloracetic, lactic, glycollic, malonic, formic, and citric acids (in the order named). They concluded that a hydroxyl and also a second carboxyl group in the acids greatly increased the swelling of the casein, but that a further increase in carboxyl groups diminished dilation. With the exception of formic acid, the monobasic fatty acids showed little swelling power.

Sutermeister (*31, p. 128*) lists a wide variety of solvents for casein, chiefly inorganic. In addition to the commonly used alkalies, he records less commonly used alkaline solvents such as sodium sulphite, aluminite, arsenate, and citrate, and lithium, magnesium, and cesium hydroxides.

Von Weimarn (*36, 37*) formed dispersions of nitrogenous materials in solutions of $Ca(CNS)_2$, $CaBr_2$, and LiCNS. The last was by far the most efficient solvent. He observed also that solutions of pyrogallol, resorcinol, thiourea, and guanidine thiocyanate were good dispersing agents—particularly at 108° C.

In a fundamental study of organic solvents for proteins, Loiseleur (*22, 23, 24*) pointed out that they formed true solutions in certain acids, such as formic, while higher acids, such as acetic and propionic, did not bring about solution. However, oxypropionic acids such as lactic or pyruvic readily dissolved casein. Other acids, such as acetic, that did not have the power of dissolving protein acquired this property when amino acids, such as glycocoll or alanine, were added.

In 1932, Soff (*29*) at Columbia University worked with new types of solvents developed by the senior author. These are concentrated aqueous solutions of highly soluble salts of organic acids such as

[3] Italic numbers in parentheses refer to Literature Cited, p. 141.

sodium cymenesulphonate or sodium benzenesulphonate in which the organic part is a large proportion of the molecule. Such solutions differ from water in their solvent characteristics, as the organic portion of the compounds seems to give them many of the properties of organic solvents. In general, Soff worked with highly concentrated solutions. He found sodium cymenesulphonate solution to be a very powerful solvent for casein and gelatin. He also stated; "There had apparently been no change in properties during storage of these casein solutions over an unusually long period of time." Nearly neutral solvents such as these salts obviously have a greater advantage in avoiding the factor of hydrolysis over stronger, more commonly used basic or acidic compounds.

Associates of Rogers (*2, p. 53*) explain that casein is insoluble in the usual neutral organic solvents and in cold dilute acids. Casein at its isoelectric point of 4.6 is considered to be uncombined with acids or bases.

EXPERIMENTAL PROCEDURE

Materials

Preliminary experiments were made to determine what concentrations would be practical to use for exact solubility work. For these preliminary experiments, sodium cymenesulphonate from the Columbia University chemical engineering laboratory was used. This was fairly pure, but for the more precise work later, the salt was recrystallized from alcohol.

Solubility determinations were run at a pH of 4.6, the isoelectric point of casein, so that the solubility would not be affected by possible alkalinity of the solvent, and also because casein is most stable at its isoelectric point. To adjust the solutions to this point, cymenesulphonic acid was used. This was purified by freeing it from sulphuric acid by the following procedure. The solution was diluted, neutralized with barium hydroxide, and the barium sulphate was filtered off. The cymenesulphonic acid was liberated from its barium salt in the filtrate by the addition of the calculated amount of sulphuric acid. After the barium sulphate was filtered off, the filtrate was evaporated to dryness.

The casein used for the preliminary experiments was a well-washed grain-curd product prepared at the Grove City (Pa.) Creamery. For the later more exact experiments, a purified casein prepared by E. O. Whittier of the Division of Dairy Research Laboratories was employed. This was precipitated from skim milk by adding a 4-percent solution of hydrochloric acid until the pH value was 4.1. The casein was washed first with water slightly acidified with hydrochloric acid, then 8 or 10 times with pure water, next with alcohol, and finally with ether. Thus the redissolving in alkali (which is a concomitant of the Hammarsten process) was avoided. This gave a casein of high purity, containing less than 0.50 percent ash above that due to the P_2O_5 combined in the casein molecule. Hammarsten casein also was used in some of the later experiments.

Methods

The general procedure for determining the solubility of casein in the solutions of the salts of organic acids was as follows: The quantity

of salt necessary to give the desired concentration of solution was dissolved in water, enough of the acid or alkali (as the case might be) corresponding to the salt used was added to bring the pH value of the solution to 4.6, and water was added to bring the whole mixture up to 55 cc. At first the casein was added gradually, often in amounts greater than 5 g, but it was found (in agreement with earlier work on solutions in other solvents) that the solubility increased with increasing amounts of the solid phase. This fact in itself was one proof of the nonhomogeneity of casein. Because of this characteristic, it was considered advisable in all cases to use the same gross quantity of casein. Consequently, the solubility values in this paper, unless otherwise noted, have been determined on the empirical basis of 5 g of casein to 50 cc of solution.

In some of the earlier experiments, much difficulty was caused by bacterial decomposition of the solutions. The addition of 0.2 percent of phenol (0.10 g) to 50 cc of solution served effectively to inhibit this action, and it was determined by experiment that phenol at the low concentration employed did not appreciably affect the solubility values.

Five grams of casein and 50 cc of solution at a pH value of 4.6 were put into a 250-cc Erlenmeyer flask. This was well stoppered and placed in an incubator that was held at the necessary temperature by thermostatic control. From two to four determinations were made in establishing the solubility value at each temperature and concentration. The flasks were shaken at frequent intervals.

The progress toward solubility equilibrium was followed by obtaining the refractive index of the solution at suitable intervals. Samples were drawn off by vacuum through a short piece of rubber tubing fitted on the end of a pipette. The tubing contained a wad of cotton to serve as a filter. An Abbé refractometer, with prisms heated to 30° C., was employed, and therefore samples of only 1 or 2 drops of solution were required. The attainment of equilibrium was shown by the constancy of the maximum of the refractive indices over a period of from 2 to 4 days. This was, incidentally, evidence against hydrolytic decomposition of the protein.

After the equilibrium of the solution was established, some of it was filtered as quickly as possible through a wad of cotton placed in the stem of a funnel. From 1 to 2 g of the solution was weighed in a weighing bottle, transferred to a beaker, and diluted with water at a temperature of 35° to 40° C. A few cubic centimeters of 10-percent acetic acid solution were then slowly added to the warm solution to precipitate the casein. At 35° to 40°, the protein separates in a granular, easily filterable condition. The mixture was allowed to stand overnight to promote completeness of precipitation. The casein was filtered off on a Gooch crucible fitted with two pads of filter paper. The filtrate was concentrated nearly to dryness on the steam plate and diluted with water. If any casein remained in solution, it was practically all removed by this method. Sometimes small additional quantities of casein were thus obtained and were added to the main residue. The Millon or biuret test was applied to the filtrate and usually showed the absence of significant amounts of casein. The precipitate was washed free of extraneous matter. Tests for completeness of washing were made with litmus paper. In

addition, lead nitrate solution was used to test for sodium cymenesulphonate; ferric chloride solution was used to test for potassium thiocyanate; and silver nitrate solution to test for sodium benzenesulphonate.

Acetic acid was chosen as the casein precipitant because this acid, as well as trichloroacetic, has been used for years for this purpose and appears to have been thoroughly tested. Colloid chemists are of the opinion that the action is a salting-out effect. The action is in accordance with the precipitating characteristics of the Hofmeister series—a satisfactory explanation of which is still lacking. To make sure that the precipitates were free from acetic acid, they were always washed until the wash water was neutral to litmus.

In the early experiments, the quantity of precipitated casein was determined by the refractometer. At times, this procedure seemed to give accurate results, but now and then the results were erratic and the source of the error could not be located.

The direct-weight method was finally adopted as the general method for determining the quantity of casein dissolved in the solvents, as it gave more consistent results than the refractometric procedure, was more expeditious than the determination of nitrogen content, and was sufficiently accurate. In obtaining the direct weight of casein, the precipitate on the suction filter was washed free of salts and treated with 60 to 80 cc of acetone and then with the same quantity of ether. The ether was removed by sucking air through the filter for 15 or 20 minutes. The Gooch crucible containing the washed casein was then placed in a desiccator and allowed to remain overnight. From the weight obtained, the number of grams of casein dissolved in 100 g of solvent was calculated.

Unless otherwise indicated, Whittier's purified casein was used for the final solubility determinations.

SOLUBILITY OF CASEIN IN SOLUTIONS OF RECRYSTALLIZED SODIUM CYMENESULPHONATE

Solutions containing 5 percent and 7.5 percent of recrystallized sodium cymenesulphonate were used since the high viscosities of the more concentrated solutions prevented effective sampling.

In general, the 5 percent solution was prepared as follows: 2.47 g of the salt was dissolved in water. Two cubic centimeters of phenol solution (containing 0.10 g) was added, followed by 4 drops of cymenesulphonic acid solution (containing 0.03 g) which gave the solution a pH value of 4.6, as shown by bromocresol green paper. The mixture was then made up to 50 cc. The 7.5 percent solution was prepared in the same way except that 3.70 g of sodium cymenesulphonate and 0.05 g of cymenesulphonic acid were used to give the correct concentration and a pH value of 4.6.

The refractive index of the solution was determined, and 5 g of casein was added. The flask was stoppered, placed in an incubator held at the required temperature, and shaken at intervals.

Equilibrium was ascertained by noting or observing the constancy of the maximum of the refractive index over a period of, usually, from 2 to 4 days. Ordinarily, all salts required an average of about 10 or 11 days to attain equilibrium at 15° C., 7 or 8 days at 30°, and 4 or 5 days at 45°.

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Percentages of casein were determined by the method of precipitation with 10 percent acetic acid solution, and weighing the casein directly as described above. Some of the early results were also checked by the Van Slyke gasometric micro-Kjeldahl method (*34, 35*) for determining nitrogen.[4] Results were usually reproducible within narrow limits, although in some cases they were not as close as could be obtained with crystalloidal materials. Proteins, because of their indefiniteness and the huge size of their molecules, never give as exact results as do simpler inorganic and organic compounds.

Final solubility values for all salts (except as otherwise noted) were determined at 15°, 30°, and 45° C. These were considered the most practical temperatures to employ, because of the limiting effect of viscosity. Solubility values are reported as grams of casein dissolved in 100 g of solvent. The solubility values are listed in table 1.

TABLE 1.—*Solubility of casein in solutions of salts of organic acids having a pH value of 4.6*

Kind and concentration of the salt solution	Quantity of casein dissolved in 100 g of the solvent at—		
	15° C.	30° C.	45° C.
Sodium cymenesulphonate:	*Grams*	*Grams*	*Grams*
5 percent	3.0480	5.9390	9.6753
7.5 percent	5.2999	8.0197	
Potassium thiocyanate:			
2.5 percent	1.7850	2.5089	3.4201
5.0 percent	3.5490		
Sodium benzenesulphonate:			
5.0 percent	.9778	1.9331	3.0480
10.0 percent	2.2070	2.7450	4.8202

SOLUBILITY OF CASEIN IN POTASSIUM THIOCYANATE SOLUTIONS

The potassium thiocyanate used was a chemically pure analyzed salt. Potassium thiocyanate solution was found to be an excellent solvent for casein. Experimentation established that a 2.5-percent solution of potassium thiocyanate was best adapted to the method of sampling and to the temperatures employed. A 5-percent solution could be used at 15° C., but at 30° and 45° the solution became too dense.

In general, the 2.5-percent solution of potassium thiocyanate was prepared as follows: 1.25 g of KCNS was weighed out, 2 cc of phenol solution was added, and the solution was made up to the mark in a 50-cc volumetric flask. Rather surprisingly, the solution gave at this concentration a pH value close to 4.6. The 5-percent solution was made up somewhat similarly, except that 2.5 g of KCNS was used, and 8 drops of 0.1 N KOH (containing 0.0022 g of alkali) were necessary at this concentration to bring the solution to pH 4.6.

Five grams of casein was added for each determination. Much the same procedure was followed as was used with sodium cymenesulphonate solutions, although in some cases the time required for the solution to reach equilibrium was a day or two longer. The results are shown in table 1.

[4] The authors are indebted to P. D. Watson, of the Division of Dairy Research Laboratories, for making some of the analyses for phosphorus and nitrogen.

SOLUBILITY OF CASEIN IN SODIUM BENZENESULPHONATE SOLUTIONS

The sodium benzenesulphonate used was a commercial product of high purity. It was found that 5- and 10-percent solutions were the most practical to use.

The 5-percent solution was prepared by dissolving 2.5 g of sodium benzenesulphonate in water and adding 2 cc of phenol solution (containing 0.10 g). About 0.25 cc of 0.2 N NaOH solution (containing 0.002 g) was required to give a pH value of 4.6. The volume was made up to 50 cc, and 5 g of casein was added as before. The 10-percent solution was prepared in the same way, except that 5 g of salt was used and about 0.5 cc of 0.2 N NaOH solution (containing 0.004 g) was added to give the required pH value. Equilibrium was attained normally at the customary three temperatures. These results are also given in table 1.

On plotting the solubility values for casein in the 5-percent solution it was noted that the solubility curve was practically a straight line. For the 10-percent solution, there was a slight curve in the line. It was thought advisable to check again very carefully the points on the curve; the values obtained by the recheck showed that the points were correct.

FRACTIONATION OF ACID-PRECIPITATED CASEIN

EARLIER INVESTIGATIONS

Several investigators have believed that acid casein as ordinarily precipitated from milk is not a homogeneous compound but a mixture of closely related proteins or fractions.

According to Linderstrøm-Lang (*20*), in 1885, Danilewsky and Rodenhausen extracted a substance from casein by boiling it with a 50-percent alcoholic solution and then by means of sulphur determinations they sought to prove a difference between the extract and the residue. In 1918, Osborne and Wakeman (*26*) treated casein with a 92-percent alcoholic solution and obtained a fraction which differed considerably from casein in phosphorus content as well as in other particulars.

In 1925, Linderstrøm-Lang and Kodama (*21*) in determining the solubility of casein in hydrochloric acid in the presence of sodium chloride, observed that the solubility increased with the amount of casein added, which suggested that the protein was not a homogeneous material. They apparently proved by the phase rule that there were present more than the four components, i. e., casein, water, sodium chloride, and hydrochloric acid. They succeeded in separating casein into two fractions. The possibility of decomposition by the strong acid at the temperature used was ruled out by formol titrations and by constant solubility values day after day.

Svedberg, Carpenter, and Carpenter (*32*, *33*) found by the Svedberg sedimentation-velocity method and the sedimentation-equilibrium method that Hammarsten casein appeared to contain several proteins of different molecular weight. By extracting casein with alcoholic HCl, they obtained a definite fraction which gave a molecular weight of $375{,}256 \pm 11{,}000$. Carpenter and Hucker (*5*) also found, by treating casein with acid alcohol and by fractionating it with potassium oxalate, that apparently three proteins having molecular weights of

98,000, 188,000, and 375,000 could be obtained from casein. According to Carpenter, hydrolysis of casein takes place very slowly.

In 1927–29, Linderstrøm-Lang (*20*) fractionated casein by means of alcoholic HCl at 60° to 70° C. He claimed that phosphorus-nitrogen ratios were excellent bases of differentiation between fractions. For example, in one fraction he found a P/N ratio of 0.0337 and in another fraction a P/N ratio of 0.0490. He postulated that either the casein is very unstable and easily breaks down into its components or else it consists of different molecules which may be slowly separated from each other. He favored the latter view. He found the yields of fractions were not quantitative; the correct proportions were not known. His results were reproducible, although he stated that—

> naturally such an experiment cannot be reproduced with the same degree of accuracy as solubility determinations with crystalline substances. One cannot expect that a fractionation of this kind will be able to give quite accurately identical results each time it is conducted. There is the possibility that milk from different cows can vary in its composition.

Linderstrøm-Lang concluded that casein is a mixture of colloidal molecules that form a coprecipitation system, and thus to a certain extent seem to act as a unit. He thought a study of fractionation should contribute much to the knowledge of the properties and reactions of casein.

Sørensen (*30*) made measurements of the solubility of proteins in salt solutions and found that solubility would increase with added amounts of proteins, thus indicating the presence of more than one solid phase. He assumed, however, that the proteins were not primarily mixtures of fractions, but "a complex combination of several components held together by residual valences." He presumed this compound to be easily dissociated into its components by the weak solvents employed, but he did not state whether the dissociation increased with time. If it did, this would be a powerful argument in favor of the dissociation theory.

Cherbuliez and Schneider (*6*) in 1932 concluded that casein was a mixture of inconstant composition. They used ammonium chloride as the fractionating agent. They stated that casein is very sensitive to the action of water when the pH value of the solution is above 7, but that it is probably perfectly stable in weakly acid solutions as at pH 4.6. These workers found that the components possessed different solubilities in 70-percent pyridine solutions and varied in their rennet-coagulation characteristics.

Berggren (*3*) separated casein by HCl and lactic acid and a dialysis procedure. She found P/N ratios of 0.0522 and 0.0558 and 0.0444 and 0.0215.

In 1934, Gróh (*15*) fractionally precipitated casein from its 40-percent urea solution and its solution in molten phenol by adding absolute ethanol, and from a 70-percent alcoholic ammonium hydroxide solution by adding dilute HCl. The initial fractions were further divided into subfractions. No evidences of hydrolysis were found.

Giza (*11*) separated casein by Linderstrøm-Lang's method, and found that fractions differed in phosphorus and in amino acid content as shown by spectrophotometric analysis. He also observed variations in the buffer capacities.

Jirgensons (*17*, *18*) fractionated Hammarsten casein, and precipitated the components by various concentrations of methanol, ethanol, and propanol. Results were plotted. Apparently a uniform protein will give a curve with a single maximum and minimum point, whereas a nonuniform protein ordinarily does not.

In 1935, Carpenter (*4*) postulated that the reversible dissociation of casein would take place at neutrality by dilution with a large volume of buffer solution.

Supplee (*2*, *p. 52*) brought about fractionation of casein by successive sedimentation and washing of fine casein. He found one fraction in particular to be very high in phosphorus.

In 1936, Pedersen (*27*) concluded that casein would dissociate during sedimentation and produce smaller molecules.

EXPERIMENTAL PROCEDURE AND EVIDENCES OF FRACTIONATION

As explained before, the possibility of selective solubility, or fractionation effect, was considered in the course of the solubility determinations. Primary evidence of fractionation was afforded if the refractometer showed an increased index on the addition of further amounts of casein after equilibrium had been attained with the quantity already present. This result, if obtained, was checked by further tests such as phosphorus and nitrogen determinations, formaldehyde absorptions, and solubilities in pyridine solution.

Some hint of fractionation was noticed in an early experiment with sodium cymenesulphonate solution. Seven and five-tenths grams of casein was the starting quantity, and an equilibrium refractive index of 1.3972–3 was first reached. On the addition of a second increment of 5 g of casein, the index jumped to 1.4030 in 1½ hours, indicating the extraction of material from the added casein. As is well known, if the solid phase is homogeneous, the solubility value at equilibrium will not be enhanced by the addition of further amounts of the solid phase. On the other hand, if the solid phase is heterogeneous, the quantities dissolved by the solvent may increase in proportion to the further increments of the material added, provided the solubility of the most soluble fraction has not been exceeded.

The same phenomenon (marked increase of the refractive index on addition of further quantities of casein) was noticed both with a 7.5-percent and a 5-percent sodium cymenesulphonate solution at 45° C., and a 2.5-percent potassium thiocyanate solution at 30°. In the latter case, fractionation was not confirmed by the P/N ratio determinations.

FRACTIONATION WITH 5-PERCENT SODIUM BENZENESULPHONATE SOLUTION

Grove City Casein

A 5-percent solution of sodium benzenesulphonate was examined for possible fractionating tendencies. A solution containing 2.5 g of the salt, 0.25 cc of 0.2 N NaOH solution (containing 0.002 g), and 2 cc of phenol solution was made up to a volume of 50 cc. This solution had a pH value of 4.6, and 45° C. was the temperature of the run. Two grams of Grove City casein was added at first. After 4 days the refractive index became stabilized. Two grams of casein was again added, and after 2 days the refractive index showed a second equi-

librium point, thus indicating the possibility of fractionation. One gram more of casein was added at this point, but shortly thereafter the thermostatic control of the incubator failed to function. The solution was then removed from the incubator, as it was thought that enough of the fractions were present to show tests. The solution was filtered through canvas, the residue washed free of sodium benzenesulphonate, as shown by the silver nitrate test, and finally washed with ethanol and ether. The substance dissolved in the filtrate was precipitated with 10-percent acetic acid, filtered, and washed in the same manner. The residue and the soluble material were dried in a vacuum desiccator over calcium chloride. In later experiments, the residue and the soluble material were more effectively dehydrated by washing with acetone and ether, and then drying in the oven at 75° to 80°.

Cherbuliez and Schneider (*6*) recommended a 70-percent aqueous pyridine solution as a reagent for distinguishing, on a basis of varying solubilities, different casein fractions. They also made use of the rennet-coagulation test to show the same distinction. These tests were applied to the residue and the soluble material obtained in this experiment. One-tenth gram of the residue fraction was added to 20 cc of a 70-percent aqueous pyridine solution. After 24 hours, the residue was only partly dissolved. The fraction obtained from the filtrate was similarly tested and it was found to be completely soluble in only a few hours. The rennet test was also applied to each fraction. To 0.1 g of each fraction was added 0.12 g of calcium hydroxide, 3.2 cc of water, and 0.16 cc of rennet solution. No coagulation was observed in the solution of either fraction.

TABLE 2.—*Results of fractionating different kinds of casein with different agents* [1]

Kind of casein and fraction	Phosphorus	Nitrogen	P/N ratio	Formaldehyde absorbed	Solubility in pyridine
Grove City casein:	*Percent*	*Percent*		*Milligrams*	
Insoluble residue	0.849	15.67	0.0542		Part.
Fraction from filtrate	2.22	15.61	.1422		Entire.
Whittier casein:					
Insoluble residue	.865	15.67	.0552		
Fraction from filtrate	.78	15.67	.0500		
SODIUM BENZENESULPHONATE (10 PERCENT)					
Whittier casein:					
Insoluble residue	0.844	15.62	0.0538	2.4	Slight.
Fraction from filtrate	.912	15.68	.0582	1.2	Entire.
Hammarsten casein:					
Insoluble residue	.786	15.67	.0502	4.7	Slight.
Fraction from filtrate	1.87	15.65	.1197	3.9	Entire.
SODIUM CYMENESULPHONATE (5 PERCENT)					
Hammarsten casein:					
Insoluble residue	0.785	15.67	0.0502	4.3	Slight.
Fraction from filtrate	1.21	15.62	.0774	1.7	Part.
Whittier casein:					
Insoluble residue	.907	15.64	.0576	5.4	Part.
Fraction from filtrate	.839	15.65	.0536	2.8	Slight.

[1] Results listed were calculated on a dry, ashless basis.

Phosphorus and nitrogen analyses were made on the two fractions. The results are shown in table 2.

The phosphorus-nitrogen ratios thus offer strong evidence that the sodium benzenesulphonate solution has fractionated the casein into components of widely differing phosphorus percentages. This evidence was confirmed refractometrically and by pyridine solubility tests.

Whittier Casein

The preceding experiment was repeated in the same way except that 5 g of Whittier casein was used. After completion of the run, the residue was washed with water, acetone, and ether. Acetone was substituted for the ethanol used in the preceding experiment because of the possibility that some alcohol-soluble component might be extracted, and also because of the statement by Cherbuliez and Schneider (*6*) that acetone helps to produce a powdery product that does not stick to the filter. The fraction in the filtrate was precipitated with 10-percent acetic acid at 35° to 40° C. The precipitate at this temperature is granular and easily filtered. At lower temperatures, a fine product is produced which clogs the filter. At higher temperatures a gummy substance is formed which cannot be washed effectively. The fraction from the filtrate was washed in the same way as the residue, and then both fractions were dried overnight in the oven at 75° to 78°.

Phosphorus and nitrogen determinations were made on these fractions. Nitrogen values were obtained by the Kjeldahl-Gunning method. Blanks were run to determine the purity of the reagents. The factor used for converting nitrogen to protein was 6.38. Phosphorus was estimated by a method for analyzing phosphorus in casein previously adapted by the authors from the methods of Meigs, Blatherwick, and Cary (*25*) and Fales (*10, pp. 213–228*).

The analytical results of this second fractionation are shown in table 2.

Some fractionation is thus indicated, although not as much as when Grove City casein was used, even though this experiment was run for a shorter time.

Different caseins vary in their proportions of fractions, according to the writers' observations and also those of Linderstrøm-Lang (*20*).

Fractionation with 10-Percent Sodium Benzenesulphonate Solution

Whittier Casein

Twenty-five grams of sodium benzenesulphonate, 2.5 cc of 0.2 N NaOH solution, and 0.5 g of phenol were made up to 250 cc of solution. This had a pH value of 4.6. Twenty-five grams of Whittier casein was added, the solution was placed in an incubator held at 45° C., and the mixture was shaken at intervals. After 6 days, equilibrium was proved by the constancy of the refractive index at 1.3602. The residue was filtered with suction and through canvas on a Büchner funnel. Because of the larger quantities of casein used, filtration by this method was extremely slow, requiring fully 3 weeks. The fraction in the filtrate was precipitated with 10-percent acetic acid solution. Both fractions were washed with water, acetone, and ether, and dried in the oven at 75° to 80°.

The residue and the fraction precipitated from the filtrate were analyzed for nitrogen by the Kjeldahl-Gunning method, and for phosphorus by the method described on page 135. The values are presented in table 2.

Some fractionation was again obtained. However, it is evident that partial qualitative but not complete or quantitative fractionation may be achieved by the use of the solvents employed. As shown later, fractionation was more definitely evidenced by the formaldehyde absorption and pyridine solubility tests.

Formaldehyde absorption.—Additional tests for fractionation were sought. It was postulated that the two fractions might vary in their affinity or absorbing capacity for formaldehyde. This would imply a difference in the number of free amino groups in each fraction, since formaldehyde is generally considered to react with the casein molecule as follows:

$$RNH_2+HCHO\rightarrow RN:CH_2+H_2O.$$

A test of the applicability of this reaction was carried out by exposing the fractions in a desiccator to the vapors of a 37-percent formalin solution. The fractions increased in weight at different rates, but since water vapor was absorbed from the formalin solution along with formaldehyde, a second variable was introduced which affected the reliability of the results.

Later, experiments were run in which the formalin solution was replaced by solid paraformaldehyde. This eliminated the water variable. The paraformaldehyde gradually gave off formaldehyde vapor which was absorbed.

The fractions were screened in order to aid in exposing equal surface areas. Those portions that passed through a No. 40 screen and were retained on a No. 60 screen, were taken for the absorption tests. Two samples of each fraction were weighed into weighing bottles and run simultaneously in the same desiccator. This was kept in the 30° C. incubator. At intervals, the dishes were removed from the desiccator, covered, and the time of covering was taken. The bottles were weighed after being allowed to come to room temperature. After each weighing, the solids in the dishes were mixed by gently shifting them back and forth in order to expose new surfaces to the action of formaldehyde. The absorption proceeded very rapidly for the first day or two, after which it advanced more slowly. Intermediate values did not always check closely, probably because of changes in exposed surfaces; but as the run proceeded, better checks were obtained until final values, after 13 days, were in close agreement. The average of checks for these final values were: For the residue, 2.4 mg; for the fraction from the filtrate, 1.2 mg. Thus the first fraction absorbs twice as much formaldehyde as the second, strongly indicating that there are more free amino groups present in the residue than in the filtrate fraction.[5] This test furnishes an additional proof of fractionation.

Pyridine solubility.—Two-hundredths of a gram each of the residue and the fraction from the filtrate were placed in stoppered test tubes, each tube containing 4 cc of 70-percent aqueous pyridine solution.

[5] It was first planned to use the formaldehyde-absorption test as a dynamic one, but intermediate rates of reaction did not check because of differences in particle size. Accordingly, only final weights of formaldehyde absorbed by the fractions were recorded.

The tubes were warmed to 40° to 45° C. for 1 hour, and then held at room temperature for 24 hours, during which time they were shaken frequently. The residue was only slightly soluble in the pyridine solution. The fraction from the filtrate was entirely soluble.

Hammarsten Casein

The preceding experiment was repeated with a different sample of casein—i. e., Hammarsten—to observe how caseins from different sources compared, and to check further the fractionating characteristics of sodium benzenesulphonate. The same kinds and quantities of materials were employed except 25 g of Hammarsten casein was used instead of Whittier casein. The conditions of the determination at 45° C. were practically the same.

Because of the extremely slow filtration resulting from the use of a Büchner funnel and vaccum filtration, a pressure filter was constructed and employed thereafter in this and the subsequent larger-scale fractionations. The pressure filter aided greatly in speeding up the time of filtration, only 9 days being required to complete this fractionation as compared to 3 weeks for the preceding one. The fractions obtained were treated by the usual procedure.

The results of the phosphorus and nitrogen analyses are shown in table 2.

Extensive fractionation is shown by the difference in the P/N ratios. Hammarsten casein is obviously fractionated to a greater degree than the Whittier casein by the sodium benzenesulphonate solution.

Not enough material was available from this particular fractionation for additional tests. Accordingly, a second experiment was carried out to obtain additional quantities of the components.

Considerably larger quantities were used this time; i. e., 50 g of sodium benzenesulphonate, 5 cc of 0.2 N NaOH, and 1.0 g of phenol were made up to 500 cc, and 50 g of Hammarsten casein was used. The action of the solvent on the casein was continued for 6 days.

The filtration of the larger quantity of residue presented much difficulty, even with a pressure filter. Parts of the mixture were finally removed and filtered by using two Büchner funnels to speed up the process. This involved some loss of material. The filtration required over 3 weeks. The fractions obtained were treated as before. When the yields were weighed, the residue was 29.40 g and the fraction from the filtrate was 1.50 g.

Formaldehyde absorption.—Screened 20-mg samples of these fractions were placed in a desiccator, over solid paraformaldehyde, and allowed to absorb formaldehyde vapors. Final values were recorded after 12 days, although absorption was still proceeding at a very slow rate. Ordinarily, 12 or 13 days is sufficient time to attain good checks and establish differences between the fractions. The final values were: For the residue, 4.7 mg; for the fraction from the filtrate, 3.9 mg.

Pyridine solubility.—Two-hundredths of a gram of each fraction was taken for the test and the same method was applied as outlined previously. The residue was only slightly soluble in 4 cc of 70-percent pyridine solution, whereas the fraction from the filtrate was wholly soluble.

The pyridine solubility test probably shows differences in the acidic groups of the fractions, whereas the formaldehyde absorption test very likely indicates differences in the free amino groups.

FRACTIONATION WITH 5-PERCENT SOLUTIONS OF SODIUM CYMENESULPHONATE

HAMMARSTEN CASEIN

Recrystallized sodium cymenesulphonate was used for this experiment. Two and forty-seven hundredths grams of salt was weighed out, cymenesulphonic acid solution equivalent to 0.03 g was added (to give the usual pH value), 2 cc of phenol was added and the solution was made up to 50 cc. Small portions of Whittier casein were poured in from time to time and the subsequent establishment of successive constant refractometric readings furnished some indication of fractionation.

The mixture was filtered, and the fractions were prepared in the regular way.

The pyridine and rennet tests were applied to these components as in preceding experiments. The fraction precipitated from the filtrate was largely dissolved in the 70-percent pyridine solution; the residue was not so soluble. The rennet test was negative in both cases. There was not enough material left for phosphorus and nitrogen determinations. Accordingly, another fractionation was attempted on a larger scale.

The solution was prepared in this way: 12.35 g of recrystallized sodium cymenesulphonate was mixed with 0.9 cc of cymenesulphonic acid solution (containing 0.1376 g per cc), 0.5 g of phenol was added, and the solution was diluted to 250 cc. It was now at the usual pH value. A 25-g sample of Hammarsten casein was used for the experiment.

Fractionation was complete in 6 days, and the solution was placed in the pressure filter. Filtration proceeded rapidly, and was finished in 3 days. The fraction from the filtrate was precipitated and both fractions were washed and dried in the customary manner. Lead nitrate solution was used to test for completeness of washing. In this test any residual cymenesulphonate will come down in white flakes as the lead salt.

Nitrogen and phosphorus values are recorded in table 2.

These values help to prove that 5-percent sodium cymenesulphonate solution is an effective fractionating agent since the P/N ratios show a wide divergence.

Formaldehyde absorption.—The residue showed a total increase of 4.3 mg, and the fraction from the filtrate, 1.7 mg. Strong confirmatory evidence of fractionation is hereby afforded.

Pyridine solubility.—The difference in solubility was not as marked in these fractions as in the fractions obtained by sodium benzenesulphonate solution. The fraction from the filtrate was evidently more soluble in the pyridine solution than the residue.

WHITTIER CASEIN

The experiment with Whittier casein was on a larger scale than the preceding ones. Forty-nine and four-tenths grams of recrystallized sodium cymenesulphonate solution was weighed out, 3.6 cc of cymenesulphonic acid solution (containing 0.1376 g per cc) was poured in to establish the correct acidity, 2 g of phenol was added, and the total mixture was diluted to 1,000 cc. One hundred grams of Whittier casein was used and the fractionation was carried on for 7 days at 45° C.

Considerable difficulty was experienced with the pressure filter in attempting to handle this large quantity of material, and some was later transferred in successive increments to a Büchner funnel. Some material was lost because of this and other difficulties. Precipitation, washing, and drying were carried out as with the Hammarsten casein. Yields were: Residue, 16.20 g; fraction from the filtrate, 36.90 g.

Phosphorus and nitrogen results are listed in table 2.

Fractionation is again indicated, but in a different manner from that in which it took place when Hammarsten casein was used.

Formaldehyde absorption.—Final weights were taken in 12 days, and were: Residue, 5.4 mg; fraction from the filtrate, 2.8 mg.

Pyridine solubility.—In this particular case it was very difficult to distinguish between fractions, although the residue appeared to be slightly more soluble than the fraction from the filtrate.

DISCUSSION AND SUMMARY

The question of possible hydrolysis of the protein is often raised in connection with fractionation work. However, under the experimental conditions used in this research, the chances of any hydrolytic decomposition are nearly negligible. No evidence of such decomposition (provided a suitable preservative was used) was obtained in the course of the experimentation. The attainment of equilibrium, as shown by constancy of the refractive index, was not dependent on time as would have been the case if hydrolysis were proceeding. The fractions in the various filtrates were always coagulable by acids. Even assuming the presence of any hydrolytic products, most of them would have been leached out by the washing liquids when the fractions were washed, and so would not affect the phosphorus-nitrogen ratios or other tests. Furthermore, the experiments were run at the isoelectric point of casein, a pH value of 4.6, the point at which there is the greatest stability and the least chance of decomposition.

The work of other investigators revealed no evidence of hydrolysis during fractionation even when much stronger reagents were used. (See Linderstrøm-Lang (*20*) and Kondo (*19*)). Soff (*29*) found no indications of hydrolysis even after long storage of casein that was dissolved in the sulphonate solutions.

SOLUBILITY OF CASEIN

From the solubility values tabulated in table 1, it will be seen (on the basis of the solubility determined at each of the three temperatures) that in almost all cases solubility increased with the temperature as a straight-line function. With a 10-percent solution of sodium benzenesulphonate, a slight curve was obtained. In the case of a concentrated solution like this, however, some minor deviation from an ideal solubility curve is not unexpected. Increased solvate formation with the sulphonate may be a factor. It will be observed that the determinations generally intercheck, i. e., they rise in a straight line and increase in definite increments between temperatures.

The solubility of casein (on the basis of 5 g to 50 cc of solution at a pH value of 4.6) in a 5-percent aqueous solution of sodium cymenesulphonate at a constant temperature of 15°, 30°, and 45° C. has been

determined. For 7.50-percent solutions, values at 15° and 30° only could be obtained. The solubilities shown by these determinations were a straight-line function of the temperature.

In the same way, the solubility of casein in a 2.5-percent solution of potassium thiocyanate at 15°, 30°, and 45° C. has been established. For a 5-percent solution of potassium thiocyanate, only the solubility at 15° C. could be determined. For the 2.5-percent solution, the solubility increased with the temperature, and was plotted as a straight line.

The solubility of casein has also been determined in 5-percent and 10-percent sodium benzenesulphonate solutions at 15°, 30°, and 45° C. For the 5-percent solution, the solubility increased in a straight line in the same manner as with the other solvents; but for the 10-percent solution, the solubility showed a slightly curved line, as previously explained.

Potassium thiocyanate solutions were found, for the first time, to be solvents for casein.

The use of the formation of lead cymenesulphonate and silver benzenesulphonate as tests for completeness of washing was developed.

FRACTIONATION OF CASEIN

It has been shown for the first time that sodium benzenesulphonate and sodium cymenesulphonate solutions at a pH value of 4.6 will separate casein into at least two components. These components, or fractions, exhibited chemical differences in that their percentages of phosphorus varied; their formaldehyde-absorbing capacities differed (probably indicating unlike numbers of free amino groups); and their solubility values in pyridine solutions varied (evidently showing differences in acidic constituents).

Potassium thiocyanate solution was not a fractionating agent.

Salts of nearly neutral action, such as these sulphonates, should be of great general value for fractionation purposes since the possibility of hydrolyzing proteins is reduced to a minimum. Emphatic criticism of the use of strong acids and alkalies as fractionating reagents has been voiced by some protein chemists because of the likelihood that the molecules may be broken down by such drastic treatment. The use of the nearly neutral sulphonates should obviate any such contingency.

It appears from an inspection of table 2 that qualitative fractionation has been achieved with these agents, but not quantitative fractionation. Strong evidences of fractionation were supplied in the experiments with the Grove City casein and the Hammarsten casein, but when Whittier casein was used the indications of fractionation were not so pronounced. This may have been due to differences in the caseins themselves, or in the methods of preparation. Svedberg, et al. (*32*, *33*) stated that the molecular weight and homogeneity of casein depended on the way in which it had been prepared. There is a possibility that partial pre-fractionation took place during the preparation of the Whittier casein as it was first washed with dilute HCl. (See Linderstrøm-Lang's procedure, p. 132.) The casein was also washed with water in tall cylinders for a considerable period of time, and some of the high-phosphorus fraction might have been separated by sedimentation. (See Supplee's method, p. 133.) Linderstrøm-

Lang (*20*) commented on the diverse ratios of the fractions in casein from different sources. Possibly, too, Sørensen's theory of the reversible dissociation of casein (*30*) might explain the lack of fractionation to uniform definite limits in all cases, since the degree of dissociation might vary with many factors involving the casein and reagent used. It will be observed that, when Whittier casein was fractionated with a 5-percent sodium benzenesulphonate solution, the P/N ratios showed an opposite trend from those obtained when fractionation was with a 10-percent solution. This may be due to differing selective solubilities of various concentrations of the salt; or if Sørensen's theory is correct, to the differing dissociating characteristics of the 5-percent and 10-percent solutions.

A new method of testing for differences in fractions has been developed which proves that the amounts of formaldehyde absorbed from paraformaldehyde by the components vary considerably. Results evidently indicate that the fractions are unlike in free amino group contents.

The pyridine solubility test has been employed as a third confirmatory method to further establish the fact of component separation. Differences in the solubility of the fractions are probably due to variations in the acidic groups present.

The refractive indices of the solutions also furnished indications of fractionation; the indices increased beyond previous equilibrium points on the addition of successive increments of casein.

The theory that casein is a mixture or a composite of loosely bound aggregates, rather than a compound, receives emphatic substantiation since a fractionation into constituents has been effected by the nearly neutral, nonhydrolyzing salts of organic acids. The fact that fractionation equilibrium was not dependent on time (as shown by the refractometer) lends evidence to Linderstrøm-Lang's casein-mixture theory rather than to Sørensen's casein-aggregate hypothesis. A further investigation of the fractions obtained should clarify even more the composition and structure of casein.

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RELATION OF BITTERNESS TO THE TOXIC PRINCIPLE IN SWEETCLOVER[1]

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INTRODUCTION

During the last three decades, sweetclover in North America has risen from the status of a roadside weed to a place of importance as a forage crop. This rapid adoption as a cultivated crop has taken place in spite of several undesirable characteristics. One of the factors at present limiting its usefulness is its rather bitter taste. Although livestock, at first reluctant to eat it, become accustomed to the taste, the bitterness may be a disadvantage when sweetclover is used for pasture in mixtures with grasses and other clovers. Another limiting factor is that unless sweetclover hay is thoroughly cured, certain changes may take place during storage in the stack or mow which render the hay toxic when fed to livestock. The resulting disease has been referred to as "sweetclover disease." Some studies on the relation between the two undesirable characteristics—bitterness and toxicity—are described in this paper.

THE SWEETCLOVER DISEASE

In 1922 Schofield (*9*)[3] called attention to a disease in cattle characterized by low clotting power of the blood and the development of extensive hemorrhages which were usually fatal. He showed the causal relation of the feeding of spoiled sweetclover hay or silage. Since that date, numerous reports of the occurrence of the disease have appeared. A careful series of studies on the pathology and therapy in cattle was made by Roderick and Schalk (*8*) with observations on the malady in other livestock. Roderick (*7*), working on cattle, has shown that in this disease the low clotting power of the blood is the result of a deficiency of prothrombin—one of the constituents of the blood essential for clotting. This finding has recently been confirmed by Quick (*5*) on rabbits by the use of the technique of Quick, Stanley-Brown, and Bancroft (*6*) for prothrombin determination.

Cannon and Greenwood (*4*) observed in rabbits a lowering of the calcium content of the blood on a diet of well-cured sweetclover hay and suggested that hemorrhage in sweetclover disease may be linked

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[3] Italic numbers in parentheses refer to Literature Cited, p. 153.

with the decline in serum calcium. In testing this point with cattle, Brown, Savage, and Robinson (*3*) found that variations in blood calcium and phosphorus were not significant either when the animals were fed well-cured sweetclover hay or when the clotting power of the blood was low following a feeding period on a known toxic sweetclover hay.

MATERIALS AND METHODS

In the present studies rabbits were used exclusively. The rabbit consumes a small amount of feed, and as was first noted by Schofield (*10*), gives a prompter response than cattle. The progress of the disease has been followed by recording the clotting time of small samples of blood taken at intervals during the feeding period. Approximately one-half cc of blood is allowed to drop from a freely bleeding incision in the marginal vein of the ear into a 50-mm watch glass. Another watch glass of the same size is inverted over the first and the time required for clot formation is determined by tilting the watch glasses at 30-second intervals at a laboratory temperature of 23° to 24° C. In a normal rabbit a firm clot forms in 5 to 7 minutes. Young rabbits, 8 weeks old, are much more susceptible than mature animals, but among rabbits of the same age a marked difference in reaction to the toxic principle has been observed.[4] Some show a prolonged clotting time with a small amount of toxic hay, while others show no symptoms even on an exclusive diet of toxic hay over a prolonged period. Further, as Roderick (*7*) found to be the case with cattle, there is no indication in rabbits of an acquired resistance to the toxic principle. One can induce the symptoms of the disease in a susceptible rabbit, allow it to recover on a nontoxic diet, and in subsequent feeding of the same amount of toxic hay the reaction will be very similar, allowance being made for advancing age. For comparisons of the relative toxicity of spoiled hays, a group of rabbits is subjected to a preliminary feeding test on a known toxic hay in amounts proportional to the body weight (45 g per 1,000 g of body weight daily) and those showing symptoms promptly in the blood clotting test are transferred to nontoxic feed in an early stage of the disease. After an interval of several days, they may be used in comparative trials.

Toxic hay can be prepared by drying to 40 or 50 percent moisture freshly cut common sweetclover, stacking it, and covering the piles as described below for individual tests. In the experiments of Roderick and Schalk (*8*), the hay was allowed to remain in small piles for 60 days or more. The writers have found, however, that the hay may become toxic in a much shorter period. In one test, 290 pounds (green weight) of Hubam, an annual form of *Melilotus alba* Desr., cut when in midflower, was dried in the sun to 49 percent moisture, put in a pile in a basement laboratory, and covered to a depth of about 6 inches with slightly wilted third-cutting alfalfa. After 8 days the pile was opened and a feeding test begun with two mature susceptible rabbits. After 7 days of feeding, one animal showed a clotting time of 30 minutes as compared with 5 minutes at the beginning of the test, while the other showed 26½ minutes as compared with 6 minutes, indicating a fairly toxic sample of hay. Another larger pile (400 pounds green weight) was found to be highly toxic in a feeding test beginning 9 days after stacking. No contin-

[4] SMITH, W. K. THE ALLEGED PROTECTIVE ACTION OF ALFALFA AGAINST THE HEMORRHAGIC SWEETCLOVER DISEASE. Unpublished.

uous temperature records have been taken in piles of sweetclover spoiled under the conditions described above, but observations indicate that after 5 to 7 days a maximum temperature is reached. The pile then begins to cool. These observations suggest the relation of the heating period to the development of toxicity.

EXPERIMENTAL DATA

TESTS TO DETERMINE TOXICITY OF SPOILED MELILOTUS DENTATA AND HUBAM SWEETCLOVER

The bitter taste characteristic of common sweetclover seems not to have been reported in any other cultivated forage crop of importance. Likewise, the disease induced by the feeding of spoiled sweetclover has not been associated with the eating of any other feed. There arises therefore the possibility of some relationship between the bitter principle of common sweetclover and the development of the toxic principle in spoiled sweetclover hay. An annual form of the typically biennial *Melilotus dentata* (W. and K.) Pers., found to be nonbitter in 1933 (*1*), seemed to offer favorable material to test the existence of such a relationship. Comparisons have been made of the toxicity of spoiled hay of the annual *M. dentata* and Hubam.

A preliminary test made in 1934 was inconclusive because of the small amount of hay available and the moldy and resulting unpalatable nature of the nonbitter hay. In this trial, however, the Hubam was found to be toxic and the nonbitter to be nontoxic.

TABLE 1.—*Reaction of susceptible rabbits when fed spoiled Melilotus dentata and Hubam sweetclover hays prepared in 1935*

Kind of spoiled hay fed	Rabbit No.	Period on diet	Weight	Period on diet	Blood-coagulation time
		Days	*Grams*	*Days*	*Minutes*
Hubam	[1] 34–R–4	0	2,170	0	5
				4	12
Do	[2] 27–A–3	0	1,910	0	5
		7	1,859	4	15½
				6	34
M. dentata	17–A–5	0	2,235	0	5
		7	2,211	4	5
		14	2,235	7	6
		21	2,213	14	6
				21	5½
Do	40–R–4	0	2,262	0	5½
		7	2,192	4	6½
		14	2,125	7	5
		21	1,819	14	5
				21	6
Hubam	[3] [4] 17–A–5	0	2,213	0	5½
		5	2,203	5	13½
Hubam plus 30 g mixed grain daily	[3] [5] 40–R–4	----------	----------	0	4
				6	7
				10	18

[1] Dead on morning of the sixth day from a typical ear hemorrhage.
[2] Died on the seventh day with severe hemorrhage into the lung cavity.
[3] 17–A–5 and 40–R–4 on Hubam after a 21-day period on *M. dentata*.
[4] Died on the seventh day with severe intestinal hemorrhage.
[5] On tenth day transferred to nontoxic feed.

In the 1935 season 400 pounds (green weight) of Hubam in full bloom and a similar amount of the nonbitter annual in early bloom were cut and allowed to dry in the field until the moisture content was 52 percent for the Hubam and 47 percent for *Melilotus dentata*.

A pile of each was made in a basement laboratory. Both stacks were covered with paper, burlap, and canvas and in both vigorous heating took place. When opened for feeding after 9 days, both hays were brown with only limited amounts of mold growth. The hays were then fed ad libitum to four rabbits (two for each pile), with the results shown in table 1.

It is apparent from the data in table 1 that the Hubam sweetclover was highly toxic, inducing a significant increase in the time of blood clotting on the fourth day and killing the two rabbits (34–R–4 and 27–A–3) on the sixth and seventh day, respectively. In contrast, the blood of rabbits on the nonbitter sweetclover (*Melilotus dentata*) remained normal over a feeding period of 21 days. At the conclusion of the initial feeding period the two surviving rabbits were transferred from *M. dentata* hay to Hubam to verify their susceptibility. Of the two rabbits, 17–A–5 consumed daily during the 21-day period amounts of *M. dentata* hay comparable to that eaten by the rabbits on Hubam and maintained its weight throughout; a blood-clotting time of 15½ minutes on the fifth day of the Hubam diet and the death of this rabbit on the seventh day (table 1) demonstrate its susceptibility. On the other hand, 40–R–4 ate somewhat less *M. dentata* than the rabbits on Hubam and lost some weight during the feeding period. To compensate for this smaller consumption of nonbitter hay, when spoiled Hubam was fed, 40–R–4 received daily 30 g of mixed grain and it was estimated that by this means the amount of Hubam ingested daily was less than the daily amount of the nonbitter hay. Nevertheless, 40–R–4 showed a clotting time of 18 minutes after 10 days of feeding, indicating its susceptibility to the toxic principle.

A quantity of hay from an additional larger stack of *Melilotus dentata* sweetclover (800 pounds dry weight) which had undergone a satisfactory spoilage was fed to two additional susceptible rabbits over a 48-day period with no indication of any change in the clotting power of the blood.

In each test, the common bitter sweetclover, when spoiled, became toxic while the spoiled *Melilotus dentata* showed no indication of toxicity. Even a low level of toxicity would have been revealed since the clotting time of the blood was determined at intervals during the feeding period. There is therefore substantial evidence that the nonbitter *M. dentata* does not become toxic when the hay is stacked with a relatively high moisture content. This finding adds weight to the suggestion that the bitter principle of common sweetclover is causally related to the development of toxicity in spoiled hay.

THE RELATION OF COUMARIN TO TOXICITY

Coumarin is responsible, in part at least, for the bitter taste of common sweetclover. It has recently been shown by Brink and Roberts (*2*) that the nonbitter species, *Melilotus dentata*, does not contain this compound in measurable amount in the vegetative tissues, although small amounts were found in the seeds. Because of the absence of measurable amounts of coumarin in *M. dentata* hay and the nontoxicity of the spoiled hay, a relationship is suggested between the presence of coumarin and the production of the toxic principle in spoiled common sweetclover hay.

Coumarin apparently does not induce the sweetclover disease. Roderick and Schalk (*8*) administered coumarin in capsules to rabbits and none of the animals showed the typical symptoms of the disease. Furthermore, large numbers of cattle and other susceptible livestock when on sweetclover pasture or on well-cured sweetclover hay ingest daily considerable quantities of coumarin with apparently no effect on the clotting power of the blood. Likewise considerable quantities of spoiled alfalfa are eaten annually by cattle with no clear evidence of the occurrence of this disease. As described in detail below, coumarin was added to alfalfa, the mixture was allowed to spoil, and the resulting product was fed to rabbits.

On October 19, 1935, a lot of 500 pounds (green weight) of freshly mown third-cutting alfalfa was spread out in a greenhouse and with frequent turning was allowed to dry to approximately 50 percent moisture. It was then put through a silage cutter, and when taken to a basement laboratory was divided into two equal portions, one of which was piled up without further treatment. The other was spread out and 1 pound of coumarin ground in a ball mill was added by sifting through an 80-mesh screen; this amount gave a concentration of coumarin of 1.4 percent on the dry-weight basis—a percentage comparable to that found in sweetclover tissue. The alfalfa was turned frequently while the coumarin was being added. It was then piled up and both lots were covered with paper, burlap, and canvas. Strong heating developed rapidly in the control pile and in 3 days vigorous mold growth was apparent. The covering was removed from this material after 8 days. The alfalfa to which coumarin had been added heated more slowly, and even after 7 days no mold growth was apparent microscopically. Because of the writers' early experience of the low palatability of moldy sweetclover, the heavily molded alfalfa was not saved for feeding. In the control pile the mold infestation was so severe that the amount of mold-free hay was insufficient for a critical test of toxicity, but in the alfalfa treated with coumarin only small amounts of moldy hay were noted towards the outside of the pile. Two susceptible rabbits, fed for 10 days on the spoiled coumarin-treated alfalfa (table 2), showed clear-cut symptoms of the disease, indicating that coumarin plays a rôle in the development of toxicity.

TABLE 2.—*Reaction of two susceptible rabbits fed spoiled coumarin-treated alfalfa prepared in 1935*

Kind of hay fed	Rabbit no.	Period on diet	Blood-coagulation time
		Days	*Minutes*
Spoiled coumarin-treated alfalfa	29–A–2	0	6
		9	17½
		10	47
Do	16–A–4	0	6
		9	41
		10	60+
Well-cured alfalfa	27–A–2	0	7
		9	6
		10	7

FEEDING OF MELILOTIC ACID AND COUMARIC ACID

It has been pointed out above that coumarin does not induce the sweetclover disease but alfalfa treated with coumarin and allowed to spoil does become toxic. There is the possibility, therefore, that during spoilage coumarin is converted into a closely related compound. Two such compounds, melilotic acid and coumaric acid, which are present in relatively small amounts in sweetclover, were prepared and each was fed in capsules to two susceptible rabbits over a period of 33 days. A mixed grain and alfalfa hay were fed ad libitum. During the first 3 days each of the four rabbits received 0.1 g of compound. The amount was increased every 3 days until on the fifteenth day each received 0.75 g and by the thirtieth day, 1.5 g. The blood of the four rabbits, which was tested at intervals of a few days during this period, remained normal throughout. If all the coumarin in sweetclover tissue were converted during spoilage into melilotic acid or coumaric acid, the amount of each would rarely exceed 1 percent of the dry tissue. An animal consuming 75 g of hay daily would not absorb an amount of each compound greater than 0.75 g daily. Further, clear-cut symptoms of the disease can be detected after 5 days of feeding on a sample of sweetclover hay of moderate toxicity. One can conclude, therefore, that the amounts of melilotic acid and coumaric acid are adequate and that neither compound is directly responsible for the symptoms of sweetclover disease.

A difference was observed between the effect of coumaric and of melilotic acid on the animals. The two rabbits receiving coumaric acid had a normal appetite during the feeding period and maintained their weight, one of the rabbits at the end of the 33-day period weighing only 34 g less than at the beginning of the period, while the other weighed 17 g more. In contrast, the animals receiving melilotic acid showed signs of digestive disturbance by the fifteenth day, consuming decreasing amounts of grain ration. Alfalfa was being fed ad libitum and no weighings were made. It was apparent, however, towards the end of the feeding period, that both animals ate only the stems and refused the leafy part of the forage. One rabbit with an initial weight of 2,665 g lost 739 g in 33 days and the other with an initial weight of 2,324 g lost 452 g.

ADDITION OF MELILOTIC ACID LACTONE TO ALFALFA BEFORE SPOILAGE

The toxicity of alfalfa treated with coumarin before spoilage has already been mentioned. Since melilotic acid occurs in sweetclover in appreciable amounts and coumarin is fairly readily converted into melilotic acid in vitro, an attempt was made to determine whether melilotic acid plays a rôle in the development of the substance or substances responsible for the diminution of the prothrombin in the blood of animals afflicted with sweetclover disease. For an adequate test an amount of acid in excess of 1 pound was required. Since the lactone form can be prepared in quantity more readily than the free acid, the former was used in this experiment.

A quantity of third-cutting alfalfa was mowed on October 6 and dried down in the field to 48 percent moisture. It was then chopped in a silage cutter, taken to a basement laboratory, and put in three equal lots, each lot containing 210 pounds of partially cured hay. The first lot was left untreated; the second received 646 g of coumarin

as described in the earlier experiment; the third was treated with 655 g of melilotic acid lactone applied to the hay as a fine spray while the hay was being turned. All lots were piled and covered as earlier described. The temperature rose in the control pile more rapidly than in the other two. The control pile was likewise the first to begin cooling off. Although no temperatures were recorded during the heating period, it was estimated that all three piles heated to a similar degree. After 14 days all piles were opened and the temperature towards the center of the pile recorded; in the alfalfa plus coumarin lot, it was 42.25° C., in the alfalfa plus melilotic acid lactone 47.5°, and in the control pile 37.5°. The portions of hay having a heavy mold infestation, which was most abundant on the control pile, less on the plus lactone pile, and slight on the plus coumarin, were discarded; the remainder—with the exception of the bottom of each pile—was spread out to dry at room temperature with circulating air. When fed to susceptible rabbits, each rabbit received hay in amounts proportional to body weight. The results of the feeding tests are shown in table 3.

The absence of toxicity on the untreated but spoiled alfalfa in table 3 is in agreement with the results of farming practice wherein annually considerable quantities of brown or moldy alfalfa are fed to cattle with no indication of the symptoms of sweetclover disease. The increase in the clotting time of the blood of the two rabbits fed alfalfa treated with coumarin confirms the results of the experiment of 1935. The association of coumarin with the alfalfa tissue during spoilage in some way leads to the development of the toxic principle. There was no indication of any toxicity in the alfalfa treated with melilotic acid lactone. Although one test cannot be considered conclusive, the fact that the hay treated with lactone was part of the same lot of chopped hay used for the test with coumarin, and after treatment was kept under conditions as nearly as possible the same as the toxic coumarin-treated material, suggests that melilotic acid lactone is not a factor in the development of toxicity.

TABLE 3.—*Reaction of susceptible rabbits fed alfalfa treated with coumarin and with melilotic acid lactone prior to spoilage*

Kind of spoiled hay fed	Rabbit no.	Period on diet	Blood-coagulation time	Kind of spoiled hay fed	Rabbit no.	Period on diet	Blood-coagulation time
		Days	*Minutes*			*Days*	*Minutes*
Alfalfa alone	53–F–5	0	7½	Alfalfa plus coumarin	S–50	0	6½
		5	6½			5	16
		7	6			7	15
Do	54–F–1	0	6	Alfalfa plus melilotic acid lactone	50–S–3	0	6½
		5	6			5	6
		7	6½			7	6
Alfalfa plus coumarin	53–F–6	0	6	Do	S–48	0	6
		5	13			5	6
		7	12			7	5

DISCUSSION AND CONCLUSIONS

The widely accepted view that the characteristic bitterness of the common sweetclovers is basically attributable to coumarin has been strengthened by the discovery of a nonbitter *Melilotus* species of

which the vegetative parts are coumarin-free. It is now shown that the presence of coumarin in sweetclover has an even wider significance. Not only does the substance contribute to unpalatibility but it also gives rise to the toxic condition in spoiled hays associated with the so-called sweetclover disease in livestock.

Two lines of evidence are brought forward showing that the disease-inducing property of certain sweetclover hays results from the coumarin present in the fresh material. Recognition of *Melilotus dentata* as a sweetclover whose vegetative parts are free of coumarin provided the basis for one approach to the toxicity problem. Supplementing the evidence from this source are the results of experiments involving the addition of coumarin to partially cured alfalfa hay.

The toxicity of the various diets employed was measured by the change in time required for blood clotting in rabbits shown to be susceptible by a preliminary test.

Using hays prepared in two different years under conditions favorable to vigorous heating, direct comparisons were made between the disease-inducing capacity of an annual form of common white sweetclover and annual *Melilotus dentata*. The evidence shows that only the common sweetclover becomes toxic, the spoiled *M. dentata* hay failing to alter the blood-clotting time even with prolonged feeding. There is thus established within the genus *Melilotus* itself a parallel between the presence of coumarin and the capacity to become toxic. It would appear from these results that the absence of coumarin in the vegetative parts of *M. dentata* is the explanation both for the nonbitterness of the species and the nontoxicity of its spoiled hays.

Confirmation of this view is found in the results of further feeding experiments in which alfalfa was treated with coumarin. Roderick and Schalk (*8*) have already shown by feeding trials with rabbits that coumarin as such is not toxic, a finding which is in accord with the general observation that cattle ingesting considerable amounts of the substance on sweetclover pastures do not develop the disease. Nor is the feeding of spoiled alfalfa hay known to affect the clotting power of the blood. In the present experiments a markedly toxic preparation was obtained, however, by adding coumarin to alfalfa dried to approximately 50 percent moisture and allowing the mixture to heat. The time required for the blood to clot increased from 6 minutes to 47 and 60+ minutes, respectively, with the two rabbits fed for 10 days on this material. This result establishes a direct relationship between the presence of coumarin and the development of toxicity in a spoiling hay.

The factor immediately responsible for sweetclover disease remains to be worked out. It is shown in the present investigations that the toxicity may arise within 7 days in a vigorously heating sample of common sweetclover hay. The inference is that some change involving coumarin occurs during the fermentation period which gives rise to a substance in the hay having a destructive effect on prothrombin in the animal. Melilotic acid and coumaric acid are found to be nontoxic so that a transformation of coumarin into either of these closely related compounds during the heating period would not explain the disease-producing property of the spoiled hay. It was likewise found that the addition of melilotic acid lactone to alfalfa preceding spoilage failed to render the hay toxic. As a working hypothesis

it may be suggested that coumarin interacting with another constituent or constituents of the plant tissue common to both sweetclover and alfalfa, under moisture and temperature conditions favorable for spoilage, gives rise to a specific toxic substance.

These findings are of significance in the improvement of sweetclover as a forage plant. The evidence clearly points to the conclusion that bitterness and toxicity have a common basis in coumarin. Reduction of coumarin content by breeding or the discovery of coumarin-free forms offers the possibility not only of improving palatability but also of removing the hazard associated with the feeding of spoiled sweetclover hays.

SUMMARY

Melilotus alba, a bitter sweetclover, on being stacked at about 50 percent moisture and allowed to heat, gives a hay which, when fed to rabbits, induces a condition characteristic of the so-called sweetclover disease in cattle by markedly lowering the clotting power of the blood.

Parallel tests with *Melilotus dentata*, a sweetclover which has recently been recognized as being nonbitter, show that this species does not become toxic on being similarly spoiled.

Neither spoiled alfalfa hay nor coumarin appears to modify the clotting power of the blood. If, however, coumarin is mixed with partially cured alfalfa hay containing about 50 percent moisture, the mixture on being allowed to heat becomes distinctly toxic.

Melilotic acid and coumaric acid, compounds closely related to coumarin, are like the latter substance in that they do not induce sweetclover disease on being fed to rabbits.

In one test melilotic acid lactone added to alfalfa before spoilage, contrary to the behavior of coumarin, did not induce toxicity.

It is tentatively suggested that coumarin interacting with another constituent or constituents of the plant tissue, under conditions favorable for spoilage, gives rise to a specific toxic substance which is responsible for the sweetclover disease in animals.

Since coumarin appears to be a basic factor in both bitterness and toxicity, the development of nonbitter forms of sweetclover gives promise not only of improving the palatability of this plant but also of eliminating the hazard associated with the feeding of improperly cured hay.

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CYTOLOGY OF TWIN COTTON PLANTS[1]

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INTRODUCTION

The occurrence of polyembryonic cottonseeds was first reported by Harland (*3*).[2] He found that seeds with two embryos occur sporadically in several strains of cultivated American cottons. Of such pairs of embryos, some gave rise to pairs of identical diploids; others to pairs consisting of one haploid and one diploid. In sea-island cottons Harland raised 16 pairs to maturity, of which 14 were haploid-diploid twins. Harland also reported haploid-diploid twins in upland cottons and in strains of *Gossypium hirsutum*×*G. barbadense* ancestry.

Harland found that haploids from particular strains of sea-island cottons were somewhat female-fertile. One haploid was unusually fertile when pollinated with pollen from Asiatic and wild American cottons, as well as with pollen from normal sea-island cotton. Other sea-island and upland haploids were found to be sterile. None of the haploids formed viable pollen.

The writer is unaware of any reports upon the cytology of cotton twins. Statements concerning the cytology of cotton haploids appear to be limited to the following: (1) Harland's (*2*) mention of Skovsted's discovery that "man cotton" is a haploid possessing only 26 chromosomes in somatic cells, (2) Skovsted's (*4*) report of a few trisomes in a haploid New World cotton, and (3) Harland's (*3*) statement to the effect that crosses of 13-chromosome Asiatic or of 13-chromosome wild American species with both haploid and diploid sea island are apparently identical. Such phenotypic expression, in conjunction with the relatively high female fertility of particular haploids, probably indicates that these haploids form a considerable number of unreduced gametes with 26 chromosomes.

OBSERVATIONS

TYPES OF TWINS

At the Rubidoux Laboratory, Riverside, Calif., within the past 3 years, four polyembryonic cottonseeds have been recorded during germination experiments. Each seed gave rise to two seedlings. The seedlings proved to be two pairs of diploid twins, one pair of which was conjoined; one pair of haploid-diploid twins; and one a diploid, the twin of which died in the seedling stage before cytological material could be obtained.

[1] Received for publication October 5, 1937; issued August 1938.

[2] Italic numbers in parentheses refer to Literature Cited, p. 160.

DIPLOID TWINS

One pair of identical diploid twins was derived from an extra large seed occurring in third-generation selfed seeds of the red variation of Acala cotton (*Gossypium hirsutum* L., No. W64). The twins were raised to maturity. They proved to be practically indistinguishable from each other and their parent. Likewise the meiotic chromosome behavior of the twins and their parent was nearly identical and similar to that of other species of cultivated American cottons (*6*). Although the twins generally formed 26 bivalent chromosomes during the first metaphase, occasionally 1 or 2 and rarely 3 quadrivalents occurred. First anaphase disjunction was normal, except for an occasional lagging bivalent. Second metaphase plates usually contained 26 chromosomes, though rarely one plate of a microspore with 1 or 2 chromosomes less than the haploid number occurred.

The second pair of diploid twins occurred in an F_2 generation of a

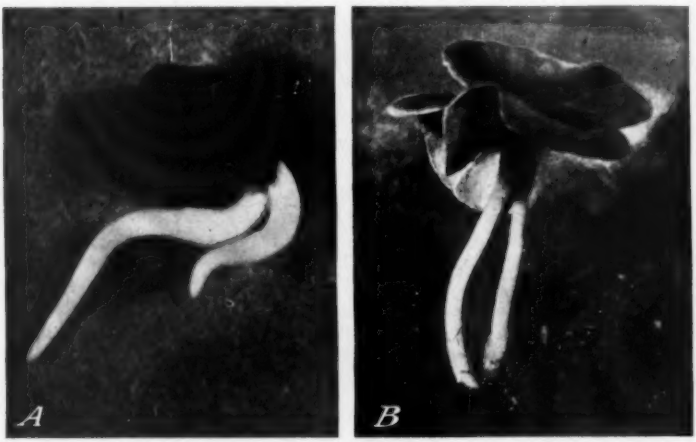

FIGURE 1.—*Gossypium nanking* × *G. thurberi*, F_2, conjoined diploid twins: *A*, Germinated seed with two radicles; *B*, young seedlings with conjoined cotyledons and hypocotyls. × 4.

hybrid between *Gossypium nanking* Meyen ($n=13$) and *G. thurberi* Tod. ($n=13$). The parent of these twins formed an average of 25.6 univalents and 0.2 bivalent chromosomes (*7*) during meiosis. It was practically sterile, forming only two open-pollinated or parthenogenic seeds. Upon germination, one of these seeds formed two radicles (fig. 1, *A*). In the cotyledon stage (fig. 1, *B*), it was evident that the seed had given rise to conjoined twins. Each of these twins had one cotyledon and shared the third, which was slightly below the other two. Their hypocotyls were united at the base of the two upper cotyledons and for fully a quarter of an inch toward the roots. As the twins grew, two buds were formed, one at the base of each free cotyledon. The buds developed into shoots approximately 2 inches long with several leaves, when the roots of the twins became diseased and the plants died.

A cytological examination of the root tips of the twins during the cotyledon stage revealed that each possessed 26 somatic chromosomes.

HAPLOID-DIPLOID TWINS

The haploid-diploid twins occurred in a culture of *Gossypium barbadense* L. (C. B.[3] No. 1065). In external appearance the seed giving rise to the twins was identical with seeds that produced only a single plant. The polyembryonic nature of the germinating seed was not determinable until the diploid twin had grown well above the soil level and its cotyledons had unfolded. At this stage of development, a second radicle appeared between the cotyledons (fig. 2, *A*), which proved to belong to a second plant. This plant was later determined to be a haploid.

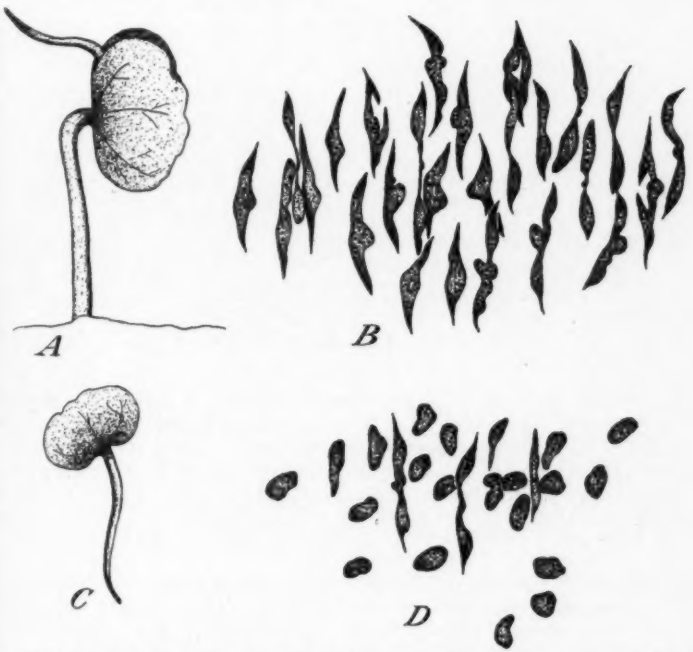

FIGURE 2.—*Gossypium barbadense*, haploid-diploid twins. *A*, Diploid twin with radicle of haploid protruding between cotyledons. × 1½. *B*, Diploid twin: First metaphase in pollen mother cell; 26 bivalent chromosomes. × 3,375. *C*, Haploid twin. × 1½. *D*, Haploid twin: First metaphase in pollen mother cell; 2 bivalent and 22 univalent chromosomes. × 3,375.

The haploid twin (fig. 2, *B*) differs from the diploid chiefly in its dwarfed stature, smaller leaves and flowers, and reduced fertility. It has been maintained for 3 years, during which it has set only three open-pollinated and one selfed seed. Attempts to produce reciprocal crosses between it and several normal cultivated American cottons have been unsuccessful.

The types of meiotic association occurring in the haploid and diploid twins and in the selfed offspring of the former are summarized in table 1. In figures 2, *B*, and 2, *D*, are shown, respectively, first metaphases of the diploid twin having 26 bivalent chromosomes, and

[3] Cotton breeding number.

of the haploid twin having 2 bivalent and 22 univalent chromosomes. Out of a group of 35 second-division pollen mother cells of the haploid, only 2 exhibited a failure of the reduction division. Each of these two cells had a single metaphase plate containing 26 chromosomes. Of the highly irregular sporads, 46 percent were tetrads and 6.6 percent diads.

The significant meiotic features are the limited chromosome pairing in the haploid, the occurrence of quadrivalents in its diploid twin, and the similarity of type of chromosomal association in the diploid twin and haploid offspring.

TABLE 1.—*First metaphase chromosome association in pollen mother cells of Gossypium barbadense*

Haploid (1065–n)			Diploid [1] (1065)			Diploid [2] (1065–2n)		
Association [3]		Pollen mother cells	Association [3]		Pollen mother cells	Association [3]		Pollen mother cells
Type	Mean		Type	Mean		Type	Mean	
		Number			*Number*			*Number*
$0_{II}+26_I$..	$0.2_{II}+25.6_I$.	22	$26_{II}+0_I$.....	$25.66_{II}+$ 0.12_I+ 0.14_{IV}	44	$26_{II}+0_I$.....	$25.64_{II}+$ 0.16_I+ 0.14_{IV}	42
$1_{II}+24_I$..		1	$25_{II}+2_I$.....		2	$25_{II}+2_I$.....		3
$2_{II}+22_I$..		2	$22_{II}+2_{IV}$.....		3	$24_{II}+1_{IV}$.....		2
			$23_{II}+2_I+1_{IV}$.		1	$22_{II}+2_{IV}$.....		2
						$23_{II}+2_I+1_{IV}$.		1

[1] Twin of haploid.
[2] Offspring of selfed haploid.
[3] The subscripts I, II, and IV are cytological symbols designating univalent, bivalent, and quadrivalent chromosomes, respectively.

The fourth pair of twins occurred in the same culture as the preceding haploid-diploid twins. The germinating seed produced two radicles, similar to those of the identical twins depicted in figure 1, *A*. One of the twins died in the seedling stage and no further study was made, except to determine that the remaining plant was a normal diploid.

DISCUSSION

Harland (*3*) has suggested that in plants originating from unlike chromosome sets polyembrony might arise as a primitive character brought into expression by genic disharmony. His suggestion is based on the fact that polyembryony in cottons has previously been found to occur only in cultivated American species that are possibly allopolyploids. All except one of the four pairs of twins recorded in the present report occurred in cultivated American cottons. That genic disharmony occurred in the parent of the exceptional pair of twins (*Gossypium nanking* × *G. thurberi*) is shown by the fact that it exhibits very little meiotic chromosome conjugation and is practically sterile. It is also noteworthy that the parental culture of *Gossypium barbadense* (C. B. No. 1065), in which occurred two of the twins recorded here, is a decidedly weak strain and somewhat sterile.

Harland further suggested that in polyembryonic cottons the occurrence of haploid-diploid twins indicates the development of two embryos, one "from an unfertilized egg alongside its fertilized neigh-

bor." This suggestion is supported by the cytological observations of Baranov (*1*), who previously reported that *Gossypium herbaceum* frequently forms ovules containing more than one embryo sac. Hence, in cases where the meiotic divisions of the parent are normal and viable pollen and eggs are formed, Harland's explanation of the origin of haploid-diploid and diploid twins is the most logical. However, in the formation of diploid *G. nanking* × *G. thurberi* twins, it appears very unlikely that 13-chromosome twin eggs should be formed and fertilized with 13-chromosome sperms. In this case it is more likely that the production of the twins involved sporophytic budding or diploid parthenogenesis.

Harland (*3*) pointed out that in cultivated cottons the degree of heterozygosity is so great that no pure lines have ever been produced. To obtain approximately homozygous strains, he suggested the use of haploids and stated: "If it were possible * * * to produce diploid shoots on the cotton haploids, we should have, ab initio, a pure line." As an alternative method of approaching homozygosity he suggested repeated matings of a standard haploid with its successive progenies of haploid × diploid. That neither of these methods is likely to produce a pure line is indicated by the chromosome pairing in the haploid and the quadrivalent formation in its selfed offspring, here reported. Observations of Skovsted (*4*, *5*) and Webber (*6*) have shown that at least four chromosomes within the haploid set of all cultivated American cottons are to some extent cytologically homologous. It is possible that these chromosomes cause haploids to be partly heterozygous and add to the complexity of the heterozygotes.

The limited pairing of chromosomes within the haploid, here reported, strongly supports Skovsted's (*5*) conclusion that cultivated American cottons are allopolyploids.

SUMMARY

The following polyembryonic plants are described: (1) Diploid twins of *Gossypium hirsutum*, (2) conjoined diploid twins of *G. nanking*×*G. thurberi* F_2, and (3) haploid-diploid twins of *G. barbadense*.

The cytology of the twins supports the suggestion that polyembryony is an expression of genic disharmony between two unlike sets of chromosomes.

The twins of *G. hirsutum* and *G. barbadense* probably developed from twin eggs, while those of *G. nanking*×*G. thurberi* F_2, probably involved sporophytic budding.

The mean chromosome conjugation of haploid *G. barbadense* was 25.6 univalents plus 0.2 bivalent; of its diploid twin, 0.12 univalent plus 25.66 bivalents plus 0.14 quadrivalent; and of its selfed offspring, 0.16 univalent plus 25.64 bivalents plus 0.14 quadrivalent.

Reference is made to the possibility of obtaining homozygous lines from haploids.

Limited pairing of chromosomes in haploid *G. barbadense* supports the suggestion that cultivated American cottons are allopolyploids.

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